Abstract

Background: Giardiasis is an intestinal diarrhoeal illness caused by the flagellate protozoan parasite *Giardia intestinalis* (synonymous with *Giardia lamblia* and *Giardia duodenalis*). Traditionally, giardiasis has been diagnosed in patients using faecal concentration and microscopy techniques. Non-microscopy based tests available for the laboratory diagnoses of giardiasis include recent innovations in polymerase chain reaction (PCR) and immunoassays with increased sensitivity. The laboratory diagnosis of giardiasis is complicated by the intermittent excretion of the parasite and asymptomatic presentation that sometimes occurs with this infection. Clinicians may on occasion treat patients for giardiasis on clinical suspicion alone when diagnostic tests have failed to identify *Giardia intestinalis* and some of the patients do get better putting into question the performance of the diagnostic test used. At the Hospital for Tropical Diseases (HTD) in London the ova, cysts and parasite microscopy (OCP-M) is the front line test for diagnosing giardiasis.

Aim: The aim of this study was to critically analyse the performance of a commercial and a published real-time PCR diagnostic tests for their potential use as front line tests for the diagnosis of giardiasis in the clinical parasitology diagnostic laboratory at the HTD. Storage conditions that will allow the best yield of *Giardia intestinalis* DNA from stored faecal samples were also investigated in this study.

Methods: In the absence of a gold standard, a composite reference standard (CRS) of enzyme immunoassay (EIA) and rapid membrane test (RMT) was used to evaluate the performance of Primerdesign Ltd. real-time PCR kit for *Giardia intestinalis* (which detects only assemblages A and B subtypes) and

a real-time PCR assay using Verweij et al published primers (Verweij real-time PCR) which targeted the (SSU) rRNA gene. The two tests were compared with the OCP-M test in a diagnostic accuracy study using a non-probability sampling technique with consecutive samples.

Results: The Verweij real-time PCR which targeted the (SSU) rRNA gene showed a diagnostic sensitivity of 93.4 % (95 % CI: 86.2 to 97.5 %) and a specificity of 74.7 % (95 % CI: 63.6 to 83.8 %) with a limit of detection (LOD) of < 5 cysts/ml. The Primerdesign Ltd. real-time PCR which also targeted the gdh gene showed a diagnostic sensitivity of 61.5 % (95 % CI: 50.8 to 71.6 %) and specificity of 98.7 % (95 % CI: 93.2 to 99.8 %) with a limit of detection (LOD) of ≤ 114 cysts/ml. Also, with a serially diluted 1 in 10 dilutions of a known concentration *Giardia intestinalis* DNA solution, the Verweij real-time PCR produced efficiency (E) of 96 % (the slope was -3.414) with a correlation coefficient (R^2) of 0.99 and a copy number variance predominantly less than 10 % (< 10 %). The Primerdesign Ltd. had E = 100 % (the slope was -3.342), R^2 = 0.95, and a copy number variance predominantly greater than 10 % (> 10 %).

In this study, the OCP-M missed 16.5 % *Giardia* positive stool samples contrasted with 6.6 % missed by the Verweij real-time PCR. The Verweij real-time PCR therefore showed approximately 10 % increase (i.e. 16.5 % - 6.6 %) in detection rate over the OCP-M and with an estimated detection limit of < 5 cysts/ml of stool, it also correctly identified 70 % (14/20) of the discrepant cases as true positives. OCP-M identified 10 % (2/20). When sensitivities were adjusted for the Verweij real-time PCR as a result of enhancement in the detection rate of the CRS, 19.3 % (94.3 % - 75 % = 19.3 %) more positive cases were noted. The Verweij real-time PCR proved to be more robust than the OCP-M and the Primerdesign Ltd. PCR and has therefore been shown to be more suited for deployment as a first line diagnostic test than the other two index tests. Even in combination with

the OCP-M, the sensitivity remained unchanged at 93.4 %. With its high specificity, the Primerdesign Ltd. *Giardia* PCR kit may be useful for partitioning clinical history for epidemiological studies but with LOD of $\leq$ 114 cysts/ml of stool and $R^2 < 0.99$ when faecal samples are involved, it will require further optimisation for use on clinical samples. Up to the end of April 2013, a literature search showed no independent evaluation of this *Giardia* real-time PCR kit.

Storage affects molecular analyses and from the findings of this study, stool samples are best stored in industrial methylated spirit and kept at 4-6 ^{o}C if they are to be used for real-time PCR for *Giardia intestinalis* detection. Alternatively they can be stored in the freezer at -20 ^{o}C without industrial methylated spirit. Samples should however be tested within three months of storage.

Conclusion: The reason why some patients get better when they are treated empirically following microscopy negative results for *Giardia intestinalis* may be found in the fact that, in this study, the OCP-M failed to detect 16.5 % of positive cases. The Verweij real-time PCR performed better than the OCP-M and showed an improvement of 10 % in *Giardia intestinalis* detection rate. The Primerdesign Ltd. *Giardia* PCR kit requires further optimisation for use on clinical samples. The Verweij real-time PCR was more robust than the OCP-M and the Primerdesign Ltd. PCR and therefore is more suited for use as a first line diagnostic test with best results obtained when stool samples are first treated with industrial methylated spirit, stored in the fridge at 4-6 ^{o}C and tested within three months of storage.

The Verweij real-time PCR assay may be used as a standalone test for in combination with the OCP-M, there was no improvement in the 93.4 % sensitivity when it was used alone. The OCP-M, however, has the advantage of identifying the presence of other parasites.

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List of Abbreviations

Abbreviation	Interpretation
AIDS	Acquired immunodeficiency syndrome
CDC	Center for Disease Control
CE MARK	Conformité Européenne
CLSI MM3-A2	Clinical and Laboratory Standards Institute document
CPA	Clinical Pathology Accreditation (UK) Ltd.
CPCR	Conventional polymerase chain reaction
DALYs	Disability adjusted life years
DNA	Deoxyribonucleic acid
DPDx	Division of parasitic diseases
EIA	Enzyme immunoassay
ESVs	Encystation-specific secretory vesicles
gdh	Glutamate dehydrogenase gene
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HTD	Hospital for Tropical Diseases
IMS	Industrial methylated spirit
IQR	Interquartal range
LOD	Limit of detection
LSHTM	London School of Hygiene and Tropical Medicine
MCP-1	Monocyte chemoattractant protein-1
NHS	National Health Service
OCP	Ova, cyst, and parasite
OCP-M	Ova,cyst, and parasite microscopy
PASW	Predictive analytics software

PCR	Polymerase chain reaction
PHE	Public Health England
PVA	Polyvinyl alcohol
QUADAS 2	Quality assessment of studies
RMT	Rapid membrane test
RT-PCR	Real-time polymerase chain reaction
SAF	Formalin, sodium acetic acid formalin
SALP-1	Striated fiber–assemblin–like protein;
SPSS	Statistical product and service solutions
(SSU) rRNA	Small subunit ribosomal ribonucleic acid gene
STARD	Standard of reporting of Studies
UCLH	University College London Hospitals
UKNEQAS	United Kingdom National External Quality Assurance Scheme
VSP	Variant-specific surface protein
WHO	World Health Organization

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I would like to thank Professor Peter Chiodini (Hospital for Tropical Diseases, London) for supporting me in higher education and encouraging me in my professional development. The time he also took to read through my project proposal and the thesis write up is much appreciated. I must also thank Dr Spenser Polley (London School of Hygiene and Tropical Medicine) for providing me with essential materials with which to conduct this study and also providing me with invaluable technical support. I thank Dr Colin Sutherland (London School of Hygiene and Tropical Medicine) for being one of my work place supervisors and helping me through the project proposal stage of the course. I am indebted to the Trustees of the Hospital of Tropical Diseases for sponsoring me for the Professional Doctorate course. A special thank you goes to Zung To at the Special Trustees office who helped me to order all the materials I needed for the project. A thank you is also extended to David Manser, the laboratory manager and all the members of staff in the Department of Clinical Parasitology for their support in specimen collection and being second readers of some of the test results I generated. The excellent tutoring skills of the staff of University of Portsmouth involved with running the Professional Doctorate course are much appreciated as well. Recognition and gratitude must go to Dr Sally Kilburn, my academic first supervisor, for her patience, encouragement, and also for her critical appraisal of my write up of the thesis. I would also like to thank Professor Graham Mills, my academic second supervisor for his critique of the research proposal, reading through my thesis draft, and general guidance throughout the duration of this course. Finally, I give thanks and praise to the Almighty God by whose Grace I was able to enrol and complete this course.

Dissemination

Even though not the direct outcome of this doctoral project, the four activities listed in Section 6.3 were works arising solely as a result of my enrolment on the Professional Doctorate programme for I had no publishing experience when I first embarked on this course.

Declaration

I declare that whilst studying for the Doctorate in Biomedical Science at the University of Portsmouth I have not been registered for any other award at another university. The work undertaken for this degree has not been submitted elsewhere for any other award. The work contained within this submission is my own work, and to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text.

Samuel Boadi

June 2013

Dedication

It gives me great pleasure to dedicate this work to my loving wife Helen and our four children: Karen, Emmanuel, Sarah, and Anne who have been a great support and encouragement to me throughout this undertaking.

Chapter 1: Introduction

1.1 Giardiasis

Giardiasis is an intestinal diarrhoeal illness caused by the flagellated protozoan parasite *Giardia intestinalis* (synonymous with *Giardia lamblia* and *Giardia duodenalis*). This chapter discusses the global burden of giardiasis and the history of *Giardia intestinalis*. Following on from these, a discussion of the biology of the parasite and the immune response that the human body produces against it is given. The remainder of this chapter will be devoted to the clinical laboratory diagnosis of giardiasis besides making a case for a more robust test to diagnose *Giardia intestinalis*.

Giardia infections have been known for many centuries and *Giardia intestinalis* has been a successful parasite because there are different genetic strains with different levels of severity and virulence factors making their elimination by the body's defence mechanisms a rather arduous task. Symptoms of infection occur when the parasite triggers a reaction. This, however, does not always occur and asymptomatic cases have been reported in the literature (Al-Mohammed, 2011; Almeida et al., 2006). Knowing that different strains exist will help in devising diagnostic tests and strategies to combat the infection. In addition to there being different genetic strains, there is also the problem of antigenic variation, whereby the parasite expresses different surface antigens so quickly that the human immune system has no time to produce antibodies against it and by that the parasite avoids detection. A variety of serological assays have been used to detect circulating antibodies in serum but because of the biological characteristics of the parasite and the lack of suitable antigens, the sensitivity of serological assays remains poor (Faubert, 2000).

The life cycle of *Giardia*, even though simple (i.e. faecal-oral), presents a diagnostic challenge in that the two stages of the parasite (trophozoite and cysts) are located in different regions in the body and a two pronged approach may be required to detect both stages. Also an added complication is the intermittent nature of excretion of the parasite. As consequence, diagnostic sensitivities rarely get to 90 % (Duque-Beltrán et al., 2002). In order to understand this protozoan parasite better, the current understanding of *Giardia* genetics and strain variation, antigenic variation, and immunological responses to the infection are discussed under sections 1.2 and 1.3. Knowing the biology of this parasite will help immensely in the diagnosis and management of this infection.

1.1.1 Global burden

Diarrhoeal diseases have been ranked second only to lower respiratory tract infections by the WHO in its 2004 update of the leading causes of global burden of disease for all ages (WHO, 1996, 2004, 2008). This represents 4.8 % of total disability adjusted life years (DALYs), a time-based measure that combines years of life lost due to premature mortality and years of life lost due to time lived in states of less than full health (Mathers, Fat, & Boerma, 2008; Yassin, Amr, & Al-Najar, 2006). Even though future prediction by the WHO puts the ranking of diarrhoeal diseases outside the top ten of the leading causes of burden of disease, it still remains an illness that requires a robust diagnostic tool in addition to public health education to keep it under control. With an estimated 280 million symptomatic human incidents per year, *Giardia* is regarded as the commonest cause of protozoan diarrheal infection worldwide (WHO, 1996).

In addition to the impact on society, there are also economic losses in the agricultural industry for *Giardia intestinalis* is also known to infect a broad range of mammals resulting in production losses (O'Handley, Buret, McAllister, Jelinski, & Olson, 2001). In fact, a study has shown that lambs infected with

Giardia parasite experienced significantly decreased weight gain, impaired feed efficiency, and reduced carcass weight compared with non-infected lambs (Olson et al., 1995). *Giardia intestinalis,* is showing no signs of abating for after a decline in UK incidence over a number of years, there has been a gradual increase in reported cases of *Giardia* infections to the Public Health England (PHE) at Colindale, UK (This was formally called the Health Protection Agency (HPA)) (Figure 1.1) (HPA, 2011)

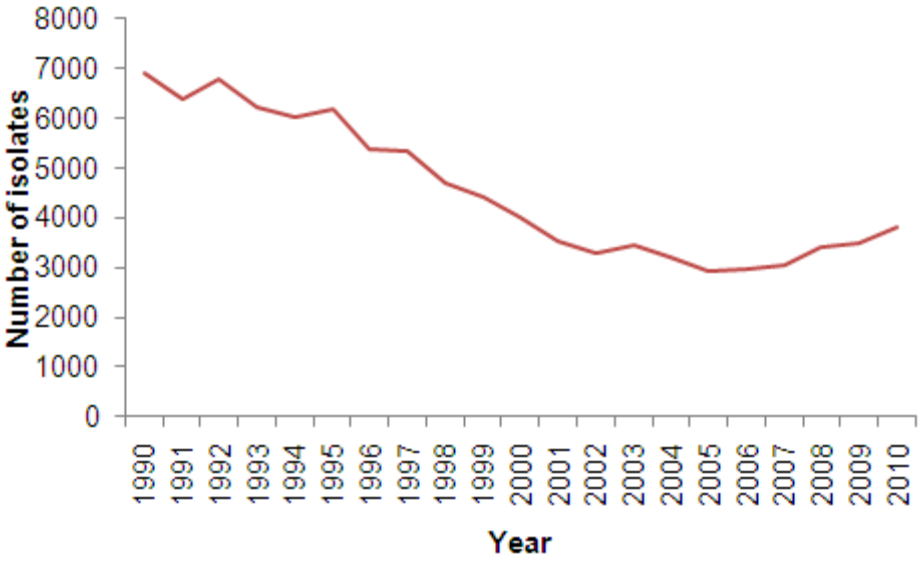


Figure 1.1: *Giardia intestinalis* laboratory reports.
All identifications reported to the Health Protection Agency England and Wales, 2000-2010. Data source: 2000-2010 Labbase2. Last reviewed: 15 July 2011.

In the same way as in the UK, giardiasis is a nationally notifiable gastrointestinal illness in the USA and during 2009-2010, the total number of reported cases increased 1.9 %, from 19,562 in 2009 to 19,927 in 2010 (Yoder, Gargano, Wallace, & Beach, 2012).

1.1.2 History of *Giardia intestinalis*

The Dutch microscopist Van Leeuwenhoek described what, most likely, was *Giardia intestinalis* in his stool when he examined it microscopically in

1681. The description of what he saw has been cited by Dobell (1920) as follows:

"*All these described particles lay in a clear transparent medium, in which I have at times seen very prettily moving animalcules, some rather larger, others somewhat smaller than a blood corpuscle, and all of one and the same structure. Their bodies were somewhat longer than broad, and their belly, which was flattened, provided with several feet, with which they made such a movement through the clear medium and the globules that we might fancy we saw a pissabed running up against a wall. But although they made a rapid movement with their feet, yet they made but slow progress."*
[Cited by (Dobell, 1920).]

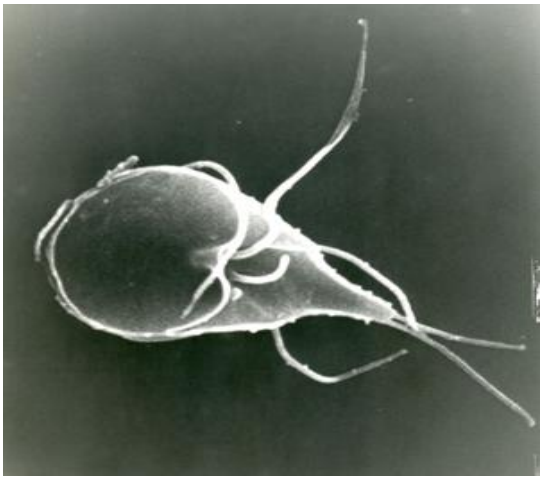


Figure 1.2: *Giardia* trophozoites under scanning electron microscope.
Credit: CDC Waterborne Disease Prevention Branch (Parasites: *Giardia* treatment, 2012)

Van Leeuwenhoek was clearly describing *Giardia* trophozoites (Figure 1.2). This flagellate was initially named *Cercomonas intestinalis* by Lambl in 1859 (Ortega & Adam, 1997) and was later renamed *Giardia lamblia* by Stiles in 1915 in honour of Professor A. Giard of Paris and Dr. F. Lambl of Prague. Many, however, consider the name *Giardia intestinalis* to be the correct name for this protozoan parasite. According to the parasitology department of the Centres for Disease Control and Prevention and for Global Health (CDC), the

International Commission on Zoological Nomenclature is reviewing this issue (Giardiasis, 2009).

When *Giardia intestinalis* was initially discovered, it was thought to be a harmless commensal organism of the gut. It was not until the 1970s when it was associated with community outbreaks and was also found in travellers returning from endemic areas with classical symptoms that it came to be considered as a pathogen. It has also been found in as many as 80 % of raw water supplies from lakes, streams, and ponds and in as many as 15 % of filtered water samples (Ryu, Alum, Mena, & Abbaszadegan, 2007).

1.1.3 Presentation of giardiasis

Giardia causes diarrhoea and sometimes malabsorption in both epidemic and sporadic forms and it is found worldwide (Ortega & Adam, 1997). The symptoms normally encountered vary, with the classical ones being: diarrhoea, abdominal cramps, bloating, and flatulence that may persist for weeks and can be intermittent or chronic. Fat absorption can be impaired giving rise to steatorrhoea. These are non-specific symptoms and giardiasis can easily be missed especially in immunocompromised and palliative care patients. Asymptomatic infections have also been reported (Al-Mohammed, 2011).

1.1.4 Transmission of giardiasis

Giardiasis is transmitted through ingestion of contaminated water and food, person-to-person contact in child care centres, and men who have sex with men. The life cycle of *Giardia* is composed of the two stages. They are: The trophozoite stage (Figure 1.3) which exists freely in the human small intestine and the cyst stage (Figure 1.3) which is the infectious form of the parasite. The cyst is relatively inert and is passed into the environment being environmentally resistant. Detection of the motile trophozoites correlates with symptomatic giardiasis. Although one can have symptoms with just cysts in the stool and both stages (independently or together) are diagnostic indicators of giardiasis.

Infections may result from the ingestion of ten or fewer *Giardia* cysts (Rendtorff, 1954).

No intermediate hosts are required. Upon ingestion of the cyst contained in contaminated water or food, excystation occurs in the stomach and the duodenum in the presence of acid and pancreatic enzymes. Two trophozoites per cyst are released and they pass into the small bowel where they multiply rapidly, with a doubling time of 9-12 h. to populate the lumen of the proximal small bowel. Here they remain free or attached to the mucosa by the ventral sucking disc until encystation is triggered as the parasite transit towards the colon. As trophozoites pass into the large bowel, encystation occurs in the presence of neutral pH and secondary bile salts. Cysts are passed into the environment, and the cycle is repeated (Figure 1.3).

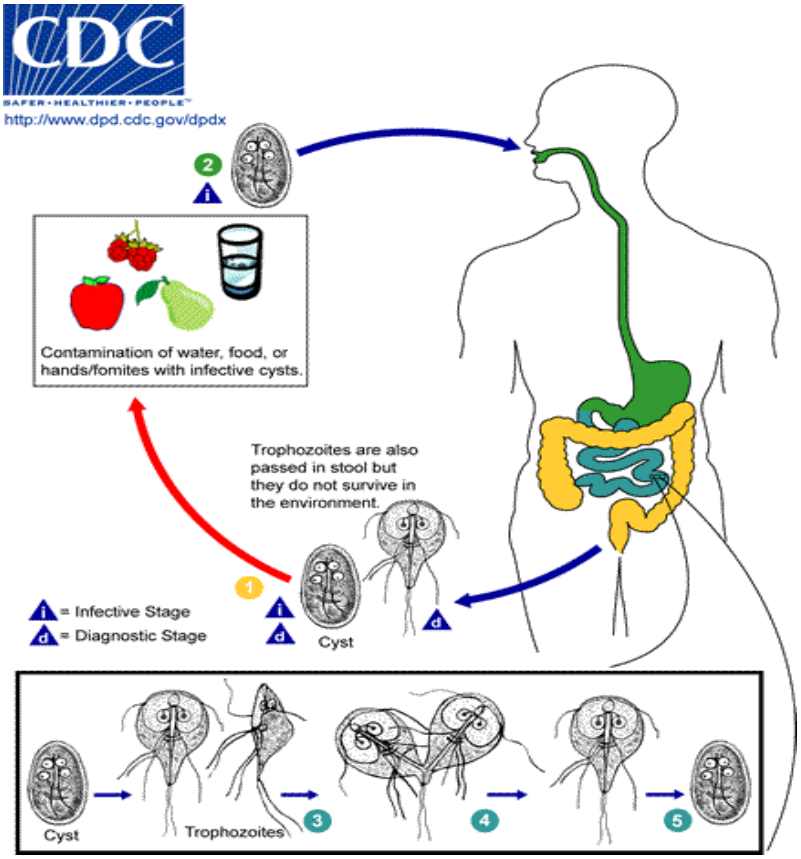


Figure 1.3: Life cycle of *Giardia intestinalis*.
Source: CDC Department of Parasitology (Giardiasis, 2009).

The cyst is the stage found more commonly in formed stools and the

incubation period is variable, 3-25 days (or longer); median 7-10 days.

The illness remains communicable as long as cysts are being shed, which may be many months. The typical shedding period is however poorly defined and may be intermittent. Humans and some animals are hosts for this parasite but human to human transmission is more common and well documented. Cattle, beaver, and other wildlife may be important in contaminating surface water supplies and domestic animals (e.g., dogs) may also be a source for some human exposures (Macpherson, 2005). In a short communication, Li et al. (2012) reported the finding of *Giardia intestinalis* zoonotic assemblage A in dogs in Guangzhou, Southern China. To prevent the potential risk of transmission to humans, they suggested the need for integrated control strategies and hygiene measures to control giardiasis (Li et al., 2012). Effective control measures cannot be put into place when diagnosis is lacking. Another group of researchers, working with Rwandan children, have associated *Giardia intestinalis* assemblage B with impaired child growth (Ignatius et al., 2012). Effective laboratory diagnosis of sub-microscopic infections is needed to clarify the actual contribution of *Giardia intestinalis* to morbidity in areas of high endemicity for these areas constitute unrecognized reservoirs of transmission (Ignatius et al., 2012).

1.1.5 Treatment of giardiasis

Several drugs have been used to treat giardiasis. Of particular note are the following five agents: nitroimidazoles, quinacrine, furazolidone, benzimidazoles, and paromomycin. The nitroimidazoles used to treat *Giardia* infections include metronidazole and tinidazole. They were discovered in 1955 and at that time found to be very effective against several protozoan parasites which included *Trichomonas vaginalis* and *Entamoeba histolytica*. It was not until seven years later, in 1962, that Darbon et al. (2001) reported the potential use of metronidazole for treating giardiasis (Darbon, Portal, Girier, Pantin, &

Leclaire, 1962; Gardner & Hill, 2001). Tinidazole is used to treat giardiasis in the Hospital for Tropical Diseases (HTD) outpatients department in London and is sometimes given as presumptive therapy for giardiasis (S.G. Wright, personal communication, June 10, 2010). The nitroimidazoles are effective against anaerobic and micro aerophilic pathogens. They exert their mutagenic effects when activated through the pyruvate:ferredoxin oxidoreductase pathway. Reduced metronidazole, for example, serves as a terminal electron acceptor which binds covalently to DNA macromolecule and thereby damages it. This eventually results in the death of the trophozoites (Edwards, 1993; Müller, 1983). A single oral dose of tinidazole has been found to be highly effective treatment for giardiasis and is equal in efficacy to a 3-day course of metronidazole (Speelman, 1985).

Quinacrine is an antimalarial drug that is also effective against *Giardia intestinalis.* Its mode of action is not fully known, it is, however, thought to intercalate readily with *Giardia intestinalis* DNA and thereby inhibit nucleic acid synthesis. Quinacrine remained the drug of choice until the early 1960s when the 5-nitroimidazole group of compounds was reported as having antigiardial activity (Escobedo & Cimerman, 2007).

Furazolidone is one of the nitrofuran compounds created since the class was discovered in the 1940s (Gardner & Hill, 2001). It is believed that its killing effect is related to the toxicity of reduced products which can damage DNA (Gardner & Hill, 2001). The benzimidazoles bind to *Giardia intestinalis* beta-tubulin cytoskeleton causing inhibition of cytoskeleton polymerization and impaired glucose uptake (Venkatesan, 1998). Two members of this class of compounds are albendazole and mebendazole. Albendazole is well known for treating helminth infections but it is also known to have anti-giardial activity (Ali & Nozaki, 2007). However, the first large-scale clinical study of albendazole, conducted in Bangladesh, showed a lower average efficacy compared with metronidazole (Hall & Nahar, 1993).

Paromomycin is a member of the aminoglycoside family, first isolated in 1956. It inhibits protein synthesis in *Giardia* by interfering with the 50S and 30S ribosomal sub-units (Edlind, 1989). *In vitro* testing shows that paromomycin has relatively low activity compared with the class of compounds mentioned above (Gordts, Hemelhof, Asselman, & Butzler, 1985).

Treatment failures have been reported with all of the common anti-*Giardia* agents including metronidazole, quinacrine, furazolidone, and albendazole. It is, therefore, important for clinicians faced with recurrence of symptoms after therapy to differentiate between actual drug resistances, cure followed by reinfection, and post-*Giardia* lactose intolerance. In this study, an attempt has been made to make this task less difficult for clinicians by discovering a more sensitive and robust test that will help with the management of this illness.

1.1.6 Prevention and control of giardiasis

PHE works to prevent and control communicable diseases. As the mode of transmission of giardiasis is via the faecal-oral route and persons remain infective as long as cysts are being shed, the illness has the tendency to go on for months in closed communities where standards of hygiene are unsatisfactory. For this reason, public health guidelines are available in the event of an outbreak to handle the situation. When the source of any *Giardia* infection is food borne, it will be recorded and reported under the heading of food poisoning by PHE. Antimicrobial treatment of individual cases forms the basis of control along with food sanitation (Salmon et al., 2004).

In the event of a suspected source of infection been identified (e.g., contaminated well or infected animal), with the potential for transmitting infection to a defined population, advice on measures to avoid exposure is given to the individuals involved. In the next section, the biology of *Giardia*

intestinalis will be discussed with particular reference to the diagnostic and potential virulence structures that characterize this parasite.

1.2 The biology of *Giardia intestinalis*

1.2.1 Trophozoite structure

Giardia intestinalis trophozoites are pear-shaped and are about 12-15 µm by 5-9 µm in size (Adam, 2001). The trophozoite has a convex dorsal surface and a flat ventral surface that contains the ventral disc, a rigid cytoskeleton composed of microtubules and microribbons. The trophozoite also contains four pairs of flagella, directed posteriorly, that aid the parasite in moving. Two symmetric nuclei with prominent karyosomes produce the characteristic face like image that appears on stained preparations.

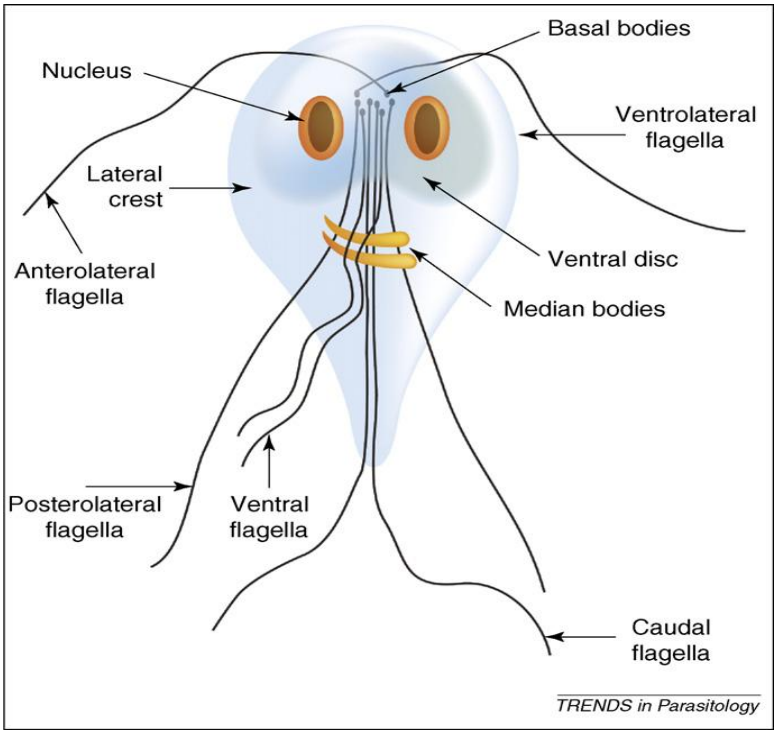


Figure 1.4: Schematic drawing of *Giardia* trophozoites. (Monis, Caccio, & Thompson, 2009).

It possesses a varied number of median bodies and it has been proposed that the median bodies play a part in the in the biogenesis of the ventral disc (Piva &

Benchimol, 2004). The location of the median bodies gives the parasite a characteristic “smile” when stained with Giemsa (Ankarklev, Jerlström-Hultqvist, Ringqvist, Troell, & Svärd, 2010). The flagella are named according to the part of the trophozoite that they are attached to. That is anterior, posterior, caudal, and ventral (Figure 1.4). They are locomotive organs, used during the excystation process and are also thought to facilitate the attachment of the ventral disc to the epithelial cell surface by creating negative pressure under the ventral disc (Piva & Benchimol, 2004). The presence of two symmetric nuclei with respect to the long axis in *Giardia* is rather unusual and it has been suggested that they may have slightly different functions because they differ in nuclei pore number and distribution (Benchimol, 2005). There is also the absence of nucleoli, which are the sites for rRNA transcription in the nuclei of higher eukaryotes. Nucleoli contain fibrillarin which is required for pre-rRNA processing. Fibrillarin has been demonstrated to be diffuse rather than localized in the *Giardia intestinalis* nuclei suggesting that rRNA transcription and processing are not localized to certain regions of the nuclei (Adam, 2001).

However, since the publication of Adam’s paper nucleolar-specific molecular markers have revealed the presence of 0.2-0.5 μm-sized intra-nuclear sub-compartment domains of fibro-granular nature at the anterior zone of both nuclei that may well indicate localized nucleoli as found in higher eukaryotes (Carranza & Lujan, 2010). The ventral adhesive disc used for attachment to enterocytes is a unique structure and covers the anterior half of the ventral side of the trophozoite. It is considered to be a virulence factor as attachment to the intestinal wall is vital for its establishment in the intestine. The known components of the disc include a family of *Giardia*-specific proteins such as α- and β-tubulin, β-, γ- and δ-giardin, SALP-1 and aurora kinase (Davids, Williams, Lauwaet, Palanca, & Gillin, 2008; Elmendorf, Dawson, & McCaffery, 2003). Some of these proteins are contractile and cytoskeletal

proteins. The ventral disc has also been suggested to play a role in nuclear division (Benchimol, 2004; Solari, Rahn, Saura, & Lujan, 2003).

Giardia has four pairs of flagella and each flagellum has a highly conserved basal body from which it emerges (Dawson & House, 2010; Sagolla, Dawson, Mancuso, & Cande, 2006). These bodies act as a type of signalling transduction and control centre during cell division and differentiation, and flagellar assembly (Davids et al., 2008; Dawson & House, 2010; Lauwaet et al., 2007). The plasma membrane of the *Giardia* trophozoite is made up of a lipid bilayer and a single coat covers the entire surface of the trophozoite. It forms the first line of defence against the innate immunity of the host. The plasma membrane also acts as the attachment point for the cytoskeleton and regulates the processes of endocytosis and exocytosis (Adam, 2001). *Giardia* trophozoites encyst as they go through the gut.

1.2.2 Cyst structure

Encystation in *Giardia* occurs after the parasite has undergone nuclear replication, but before cytokinesis. This prepares the cyst to release two trophozoites upon excystation. The mature cyst therefore contains four nuclei (Figure 1.3). The cyst is smooth-walled and oval in shape, measuring 8-12 µm long by 7-10 µm wide. Once the host is infected, trophozoites may appear in the duodenum within minutes. Excystation occurs within 5 min of exposure of the cysts to an environment with a pH between 1.3 and 2.7 (Mukherjee et al., 2011). After infection, the trophozoites attach to the enterocytes via the ventral adhesive disk. This may occur through the presence of lectin on the surface of the trophozoite or through other mechanical means. Encystation is a continuous process during infection. As the trophozoites encounter neutral pH and/or secondary bile salts, encystation-specific secretory vesicles (ESVs) appear. After 15 h, cyst wall proteins are visible. Within 24 h after the appearance of ESVs, the trophozoite is covered with these cyst wall proteins, the form of the

cyst has emerged, and new antigens are present. The cyst wall is tough and consists of 60 % carbohydrate and 40 % protein that prevents hypotonic lysis in the environment (Ankarklev et al., 2010). Three cyst wall proteins (CWP1, CWP2, and CWP3) have been identified and these are potential targets for enzyme immunoassay tests for diagnosing giardiasis.

1.2.3 Taxonomy of *Giardia intestinalis*

The taxonomy of *Giardia* is evolving and this account relates to what is currently known in this field of study. The 1980 classification of the Protozoa, based on morphology, places *Giardia* in the phylum Sarcomastigophora, sub-phylum Mastigophora (Flagellata), class Zoomastigophorea, order Diplomonadida and family Hexamitidae (Morrison et al., 2007). According to the new or current classification, which is based on genetic, structural, and biochemical analysis, *Giardia* belongs to the Phylum Metamonada, Subphylum Trichozoa, Superclass Eopharyngia, Class Trepomonadea, Subclass Diplozoa, Order Giardiida and Family Giardiidae(Cavalier-Smith, 2003; Plutzer, Ongerth, & Karanis, 2010). *Giardia* is very unusual in the sense that it is a eukaryotic single celled organism and yet shares many characteristics with anaerobic prokaryotes. It lacks common eukaryotic sub-cellular organelles such as mitochondria, peroxisomes, and a discernible steady-state Golgi apparatus (Plutzer et al., 2010). The functions of the latter appear to be taken over by other structures. For despite the lack of any morphological similarities, *Giardia* ESVs show several biochemical characteristics of maturing Golgi cisternae (Marti & Hehl, 2003).

1.2.4 Genetics and strain variation

The Genus *Giardia* currently has six species identified by morphological features using light and electron microscopy and subsequently confirmed by molecular analysis of the small ribosomal (18S) RNA gene (Adam, 2001; Plutzer et al., 2010). These species are associated with particular hosts as shown

in Table 1.1. Isolates of *Giardia intestinalis* are classified into seven assemblages, based on the characterization of the glutamate dehydrogenase (*gdh*), small-subunit (SSU) rRNA, and triosephosphate isomerase (*tpi*) genes (Bertrand, Albertini, & Schwartzbrod, 2005).

Table 1.1: *Giardia* species host associations.

***Giardia* species**	**Host**
Giardia agilis	Amphibians
Giardia ardeae	Birds (Herons)
Giardia psittaci	Birds (Psittaci)
Giardia muris	Rodents (Mice)
Giardia microti	Rodents (Voles)
Giardia intestinalis species complex	Human and other mammalian hosts

Giardia intestinalis species complex is composed of different strains isolated from a large range of mammalian hosts including humans (Cacciò, Thompson, McLauchlin, & Smith, 2005; McRoberts et al., 1996). To date, eight assemblages (A to H) are delineated within the *Giardia intestinalis* complex and H is not yet fully described. Assemblages A and B only are associated with humans and the rest with other mammalian species as shown in Table 1.2. Molecular analyses of these assemblages have shown that they are not close enough to be grouped under the same species. Sequence analysis indicates that the distances between these assemblages are actually greater than those separating certain genera of bacteria (Monis et al., 2009). Hence, the suggestion that the species complex should be broken up into different species with their own particular name assigned to them as shown in Table 1.2 (Lasek-Nesselquist, Welch, & Sogin, 2010; Lasek-Nesselquist, Welch, Thompson, Steuart, & Sogin, 2009).

Table 1.2: *Giardia intestinalis* assemblages.
Species names have been suggested for *Giardia intestinalis* assemblages.

***Giardia intestinalis* assemblage**	**Proposed species**	**Host**
A	*G. intestinalis*	Humans and other primates, dogs, cats, livestock, rodents, other wild mammals
B	*G. enterica*	Humans and other primates, dogs, cats and some species of wild animals
C/D	*G. canis*	Dogs and other canids
E	*G. bovis*	Cattle and other hoofed animals
F	*G. cati*	Cats
G	*G. simondi*	Rats
H	(New species not yet formally described)	Pinnipeds (marine mammals)

It is known that the genetic loci of *Giardia* differ in substitution rates. The substitution rates for the partial (SSU) rRNA, bg, gdh, and tpi genes have been reported to be 0.01, 0.03, 0.06, and 0.12 substitutions per nucleotide, respectively (Wielinga, Ryan, Andrew Thompson, & Monis, 2011). These differences have resulted in different resolution of parasite typing. The (SSU) rRNA has been used mostly for genotyping, whereas the most variable locus, tpi, is usually used for subtyping. The bg and gdh loci, with substitution rates between those of the (SSU) rRNA and tpi genes, have a broad application spectrum (Table 1.3) (Feng & Xiao, 2011).

Table 1.3: Commonly used *Giardia intestinalis* genotyping tools. Target, primer, and assay type (Modified from Feng &Xiao, 2011).

Gene	Primer (sequence [5'-3'])	Size(bp)	Specificity	Assay type	Usages (s)
tpi	AL3543 (AAATIATGCCTGCTCGTCG)	605	Genus specific[a]	Nested PCR, sequencing	Genotyping and subtyping
	AL3546 (CAAACCTTITCCGCAAACC)				
	AL3544 (CCCTTCATCGGIGGTAACTT)	532			
	AL3545 (GTGGCCACCACICCCGTGCC)				
gdh	Ghd1 (TTCCGTRTYCAGTACAACTC)	754	Genus specific	Nested PCR, sequencing	Genotyping and subtyping
	Gdh2 (ACCTCGTTCTGRGTGGCGCA)				
	Gdh3 (ATGACYGAGCTYCAGAGGCACGT)	530			
	Gdh4 (GTGGCGCARGGCATGATGCA)				
gdh	GDH1 (ATCTTCGAGAGGATGCTTGAG)	778	Genus specific	PCR, RFLP, sequencing	Genotyping and subtyping
	GDH4 (AGTACGCGACGCTGGGATACT)				
gdh	GDHeF (TCAACGTYAAYCGYGGYTTCCGT)	432	Genus specific	Seminested PCR, RFLP	Genotyping and subtyping
	GDHiF (CAGTACAACTCYGCTCTCGG)				
	GDHiR (GTTRTCCTTGCACATCTCC)				
(SSU) rRNA gene	RH11 (CATCCGGTCGATCCTGCC)	292	Genus specific	PCR, sequencing	Genotyping
	RH4 (AGTCGAACCCTGATTCTCCGCCCAGG)				
	GiarF (GACGCTCTCCCCAAGGAC)	130			
	GiarR (CTGCGTCACGCTGCTCG)				
bg	G7 (AAGCCCGACGACCTCACCCGCAGTGC)	753	Unknown	Nested PCR, sequencing	Genotyping and subtyping
	G759 (GAGGCCGCCCTGGATCTTCGAGACGAC)				
	GiarF (GAACGAACGAGATCGAGGTCCG)				
	GiarR (CTCGACGAGCTTCGTGTT)	511			

[a] Does not amplify assemblage D

1.2.5 Pathophysiology and pathogenesis

The host-microbial interactions that govern the outcome of infection are not fully understood. Available findings, however, show that microvillus atrophy and enterocyte injury resulting from *Giardia* infection result in intestinal malabsorption and hypersecretion (Buret, 2008). *Giardia*-induced enterocyte apoptosis causes the pathophysiological activation of CD8+ lymphocytes which in turn induces diffuse shortening of brush border microvilli resulting in malabsorption and maldigestion (Cotton, Beatty, & Buret, 2011).

Osmotic diarrhea can also occur in *Giardia* infections due to secondary lactase and other enzyme deficiencies in the microvilli (Wiser, 2007).
Nitric oxide is an important mediator of homeostasis. It is synthesized from L-arginine and any changes in its functions can affect the pathological state of an organism. It has been reported that *Giardia* spp. inhibits nitric oxide production by consuming arginine. This could contribute to the variability of the duration and severity of infections by this parasite (Pavanelli et al., 2010).

1.3 Immune response to *Giardia intestinalis*

Epidemiological studies have shown that previous infection with *Giardia* is followed by a reduced risk of re-infection and a reduced development of overt symptoms (Solaymani-Mohammadi & Singer, 2010).

1.3.1 Mechanisms of the immune response

The immune response to microbial pathogens, including *Giardia* sp., relies on both innate and adaptive components. Although the actual host defence mechanisms responsible for controlling *Giardia* infections are poorly understood, many studies have demonstrated the development of adaptive immune responses as well as innate mechanisms in humans and other animals (Gillon, Al Thamery, & Ferguson, 1982; Roxström-Lindquist, Palm, Reiner, Ringqvist, & Svärd, 2006). Long et al. (2010) looked at the role of faecal chemokines and cytokines in the resolution of diarrhoeal *Escherichia coli* and

Giardia intestinalis infections and found increased levels of monocyte chemoattractant protein-1 (MCP-1), IFN-gamma, IL-4, and IL-5 to be associated with increased *Giardia intestinalis* infection duration, while increased IL-8 levels were associated with decreased duration (Long et al., 2010). However, these associations may not represent the development of a protective immune response in the gut**.** More effort should be directed to understanding mechanisms of virulence and identifying specific parasite virulence factors in order to understand the relative contributions of both the host and the parasite to disease (Long et al., 2010).

1.3.2 Antigens and antigenic variation

Antigenic variation in *Giardia intestinalis* is the ability to spontaneously switch to a different variant-specific surface protein (VSP). It allows the parasite to evade the host's immune response and by that produce chronic and/or recurrent infections. There are approximately 190 VSP-coding genes and only one is expressed on the surface of each parasite at a particular time. The system of regulation of the VSP expression has been shown to comprise RNA-dependent RNA polymerase, dicer, and argonaute which are known components of the RNA interference machinery (Prucca et al., 2008).

1.3.3 Immune-compromised hosts

Individuals with HIV and AIDS do not appear to be at particularly increased risk of developing symptomatic giardiasis. Parasite clearance is believed to be reliant more on secretory immunity in the intestinal lumen than cell-mediated responses within the intestinal mucosa. Specific secretory (s) IgA has been detected on the surface of *Giardia intestinalis* trophozoites in human jejunal biopsies and jejunal fluid (Farthing, Cevallos, & Kelly, 2008). Anti-*Giardia* sIgA has also been found in milk and saliva and there is the suggestion that they contribute to protection from giardiasis in breast-fed infants. Current evidence suggests that anti-*Giardia* sIgA has a role in clearing *Giardia* from the

gut lumen, possibly by trophozoites agglutination and/or inhibition of flagella motility (Farthing et al., 2008). It has also been observed in clinical practice that AIDS patients, and especially those with low immunoglobulin levels, are difficult to treat when they get giardiasis (P. Chiodini, personal communication, June 14, 2012). Using an enzyme-linked immunosorbent assay to detect IgM, IgG, and IgA, specific to *Giardia intestinalis* trophozoites, Janoff, Smith, and Blaser (1988) tested sera obtained from a group of AIDS patients and healthy heterosexual men. Patients with AIDS who had acute symptomatic giardiasis had significantly lower levels of all antibodies than did the heterosexual subjects who had giardiasis; specific IgM too was absent in all but one patient with AIDS. In spite of this result, they remarked that the treatment available for AIDS patients is independent of the patient's immune status and therefore patients' with AIDS do not have to suffer from prolonged symptomatic *Giardia intestinalis* infection (Janoff, Smith, & Blaser, 1988). Another group of researchers (Cardoso et al., 2011), described the epidemiology of intestinal parasites in patients from an AIDS reference centre in Brazil. They suggested that the development of symptomatic giardiasis cannot be associated with a particular arm of the immune system (Faubert, 2000). Experiments using mice have also suggested the importance of CD4+ cells in parasite clearance by switching B-cell IgM to IgA production during infection(Farthing et al., 2008). Therefore, the suggestion that the treatment given to AIDS patients with giardiasis is independent of their immune status is yet to be substantiated.

1.3.4 Vaccination

Human vaccination against *Giardia* infection is currently not available, though a crude veterinary vaccine has been licensed for cats and dogs. Jenikova et al. (2011) tested the vaccine potential of three conserved antigens previously identified in human and murine giardiasis, α1-giardin, α-enolase, and ornithine carbamoyl transferase, in a murine model of *Giardia*

intestinalis infection. Their findings indicated that the α1-giardin is a suitable candidate antigen for a vaccine against giardiasis (Jenikova et al., 2011).

It was mentioned in Section 1.3.2 that the system of regulation of the VSP expression has been shown to comprise RNA-dependent RNA polymerase, dicer, and argonaute, known components of the RNA interference machinery (Prucca et al., 2008). Disruption of the pathway for this system of regulation of the VSP expression generates trophozoites simultaneously expressing many VSPs. The fact that the parasite uses antigenic variation for survival means that the expression of many VSPs at the same time might be a useful thing in generating vaccines against the parasite (Prucca, Rivero, & Luján, 2011; Rivero et al., 2010).

1.4 Laboratory diagnosis of giardiasis

Since the 17th century when the Dutch scientist Antony van Leeuwenhoek used a microscope to examine his own diarrhoeic stool sample, the microscope has become a scientific tool in the control and treatment of protozoal diseases. Recent advancement in technology has opened up new approaches for the development of improved diagnostic tools for the detection *Giardia intestinalis* (de Waal, 2012). The aim of this section is to highlight how these have contributed to the diagnosis of giardiasis and any further developments that may be required to improve on .

1.4.1 Microscopy

Traditionally, giardiasis has been diagnosed in patients using the faecal concentration technique described by Allen and Ridley (Allen & Ridley, 1970; Ridley, 1956). The procedures for the direct microscopic detection of cysts and trophozoites in fresh or fixed stool specimens are, however, laborious, time consuming and require expertise (Gaafar, 2011). Also, the sensitivity of parasite detection is hampered by the intermittent pattern of excretion of the parasite which means that they may be at sub-optimal levels for detection using

the direct smear or concentration method (Duque-Beltrán et al., 2002). The sensitivity of parasite identification has been reported to increase up to 85 % when microscopic examination is performed on three faecal samples obtained on different days (Duque-Beltrán et al., 2002; Gaafar, 2011). Both non-invasive and invasive procedures have been used to obtain samples for diagnosing giardiasis.

1.4.1.1 Use of non-invasive methods

Stool samples are commonly examined for the investigation of gastrointestinal disturbances. The samples are normally obtained without any invasive procedure and the simplest method of microscopic examination is the examination of smears with further staining and wet preparations with or without staining (Garcia, 1999). A direct wet preparation of a small amount of stool mixed with normal saline is prepared on a microscope slide. This is useful for the identification of *Giardia* trophozoites. The advantage is that the movement of these parasites can be observed which aids identification. The direct wet preparation can also be used to identify the cyst stage of *Giardia*. Further staining of the faecal smear with a Romanowsky-type stain (e.g. Rapid Field) and the wet preparation with iodine is often used to improve the sensitivity of this technique (de Waal, 2012). The greatest disadvantage of smears/wet preparations is their lack of sensitivity. To improve sensitivity, methods for concentrating *Giardia* cysts from a larger volume of starting material before microscopic examination have been developed. Of particular note is the Allen and Ridley method for stool concentration which is still the gold standard for microscopic examination for ova, cysts, and parasites (Allen & Ridley, 1970; Ridley, 1956). Formalin (10 %) and ether are mixed with the stool sample to remove extraneous substances like faecal fat and large faecal debris. The resulting mixture is spun down to deposit the parasites in the form of a pellet. The supernatant is discarded and the pellet is examined

microscopically after reconstituting with a couple of drops of normal saline. In a study conducted to compare formol-ether concentration techniques and direct smear for the diagnosis of intestinal parasites, the formol-ether concentration technique detected 65.3 % of positive specimens for one or more parasites including *Giardia* and the direct smear detected 34.7 % (Oguoma & Ekwunife, 2007) . The formol-ether concentration technique detects *Giardia* cysts and cysts and larvae of other parasites so in this project, the direct smear was added to detect *Giardia* trophozoites which otherwise will not be detected because the ether and formalin mixture for the concentration technique destroys the trophozoites. Even though the concentration method is relatively more sensitive, it, however, does not to detect the trophozoites stage.

1.4.1.2 Use of invasive techniques

In patients with chronic diarrhoea and negative stool examinations, the diagnosis of giardiasis can be established by the examination of duodenal contents. The patient swallows a gelatin capsule on a string (Entero-test) (Beal, Viens, Grant, & Hughes, 1970). After several hours, the capsule is removed by pulling the string up and the string is examined microscopically for trophozoites. Rarely, duodenoscopy with microscopic examination of duodenal fluid or histologic examination of biopsy specimens will be required to establish a diagnosis. Although more invasive, it has the advantage of revealing alternative diagnoses especially in HIV positive patients who have a weakened immune system and therefore are more susceptible to infections (Adam, 1991). Also Coeliac disease is looked for in biopsies. Some researchers have found the examination of duodenal contents to be more sensitive than the examination of stool specimens (Kamath & Murugasu, 1974; Rosenthal & Liebman, 1980). This is in contradiction to Goka et al. (1990) who found stool samples to be more reliable than duodenal aspirate (Goka, Rolston, Mathan, & Farthing, 1990). In another study by a different group of workers, cysts were found in the

stools of all ten experimentally infected persons, while duodenal aspirates yielded negative results (Nash, Herrington, Losonsky, & Levine, 1987). Therefore, it would seem that the evaluation of stool and duodenal contents complements each other, one test may be positive while the other is negative. Experimental infections like this raise significant ethical issues and therefore an ethical framework has been offered for evaluating infection-inducing challenge experiments, which focuses on the scientific and public health rationale, among other things, for conducting these studies (Miller & Grady, 2001). This experiment was done on healthy volunteers and extrapolating the results to vulnerable groups of people with compromised or immature immune systems will not be straightforward. Alternative approaches to this type of investigation will be much preferred, hence the need for more sensitive methods to test stool samples.

1.4.2 Immunodiagnosis

Immunodiagnostic tests use antibody-antigen reactions to diagnose infection. In direct detection, the antibodies are usually the reagents and antigens are the disease markers. If organisms occur at densities below the detection level of the direct method employed, or cannot be directly demonstrated because of the particular stage of their life cycle found in the biological sample, serological tests are used to diagnose the infection indirectly.

1.4.2.1 Antibody detection

Indirect fluorescent antibody test (IFAT) is commonly used to detect the presence of antibodies against *Giardia intestinalis* and by so doing attempt to diagnose the infection indirectly. A polyvalent conjugate (recognizing IgG, IgM and IgA) is used and a four-fold or greater increase in titre between acute and convalescent sera indicates an acute active phase. A single positive reaction represents previous exposure, since antibody titres are known to remain elevated for at least six months. A titre $\geq$ 1:32 is considered as antibody

detected, and antibody not detected is < 1:16. A titre of 1:16 is considered as equivocal and a repeat test, 3 to 4 weeks after the initial specimen, is tested if clinically indicated (Mayo Medical Laboratories, 1995). The reason behind this cautious approach to the interpretation of the IFAT results is the fact that antibodies persist for a long time, even after the elimination of the parasite and therefore a positive test does not necessary mean an active infection is going on. Serology is not useful for diagnosing acute infections (de Waal, 2012) because it takes a long time for the body to develop protective immunity against *Giardia*. Even so, only partial immunity occurs for immunity does not necessarily develop following a single infection. In a community that experienced two *Giardia* outbreaks in an interval of five years, people infected during the first outbreak were at significantly lower risk during the second outbreak (Isaac-Renton, Lewis, Ong, & Nulsen, 1994). There is also variability in the humoral response to *Giardia* infection with infants and young children particularly affected because of their immature immune system and often are exposed more to the infection than other age groups (Robertson, 1996). Antigenic variation in *Giardia* species is also one of the reasons why serology is not useful for diagnosing acute infections. The parasite changes the expression of its surface antigens more often before the body can mount a response to any trigger (see Section 1.3.2). Serum antibody testing is useful epidemiologically, but is not sensitive or specific enough for the primary diagnosis of individual patients (Goka, Rolston, Mathan, & Farthing, 1986; Smith, Gillin, Brown, & Nash, 1981; Visvesvara, Smith, Healy, & Brown, 1980). An alternative is to detect *Giardia intestinalis* antigens rather than host antibodies raised against it.

1.4.2.2 Antigen detection

Monoclonal antibodies have been raised against *Giardia* to detect the presence of the parasite. For example, The most widely used antigen detection immunoassays for *Giardia* are the direct fluorescent-antibody (DFA) test which

detects *Giardia* cysts (Garcia, Shum, & Bruckner, 1992), and enzyme immunoassays (EIAs), which detect soluble stool antigens (Garcia & Shimizu, 1997, 2000).

DFA tests (e.g., The Merifluor DFA (Meridian Bioscience, Inc.) provide definitive diagnosis by using fluorescein-labelled antibodies against cell wall antigens of *Giardia* cyst. The sensitivity and specificity of the Merifluor DFA kit has been reported as 96 to 100 % and 99.8 to 100 % respectively (Johnston, Ballard, Beach, Causer, & Wilkins, 2003). This test has greater sensitivity than traditional examination of permanent smears for *Giardia* (Mank, Zaat, Deelder, van Eijk, & Polderman, 1997).

Commercially available EIAs use antibodies for the qualitative detection of *Giardia* antigens in both preserved and unpreserved stool specimens (Rosoff et al., 1989). The reported sensitivities of EIAs range from 94 to 97 % and specificities range from 99 to 100 % (Johnston et al., 2003).

EIAs are useful when there are numerous samples to be screened because the micro-titre tray can take about 96 samples. The test can also be read objectively on a spectrophotometer as well as visually based on the level of fluorescence. However, false-negative test results have been reported (Hanson & Cartwright, 2001).

A rapid membrane test (RMT), manufactured by Coris BioConcept uses monoclonal antibodies raised against *Giardia intestinalis* cyst membrane antigens to detect the parasite. The kit is CE marked and marketed with the product name of *Giardia*-strip with reported 96.3 % sensitivity and 97.8 % specificity with performance compared to microscopy (Coris BioConcept, 2012).

Lack of trained microscopists, costly equipment, and the need to cut down turnaround times for the issuing of results have contributed to the development of immunochromatographic lateral-flow immunoassays (rapid assays) for *Giardia intestinalis.* These tests are simple, 10 min card assays with reported

sensitivity of greater than 97 % and specificity of 100 % (Chan et al., 2000; Garcia & Shimizu, 2000).

1.4.3 Detection of parasite DNA

The advent of nucleic acid-based diagnostics, in particular the polymerase chain reaction in 1985, has revolutionized the diagnosis of infectious diseases in general. Given the limitation of staining techniques including the inability to do species or genotype identification, various molecular methods have been developed to diagnose giardiasis for treatment purposes and epidemiological studies. For example, SYBR Green real-time PCR developed by Polley et al. (2011) has now replaced microscopy as the frontline test in the detection and species identification of microsporidial infections at the Hospital for Tropical Diseases in London (Polley, Boadi, Watson, Curry, & Chiodini, 2011). A number of conventional species-specific PCRs and probe-based real-time PCRs also exist for the detection of *Giardia intestinalis* (Hopkins et al., 1997; Nantavisai et al., 2007; Verweij et al., 2004; Verweij et al., 2003). One such molecular method is a real-time PCR assay by Primerdesign Ltd. which targets the gdh gene of *Giardia intestinalis* and a nested conventional PCR targeting the (SSU) rRNA gene of *Giardia intestinalis* has a reported sensitivity of 97.3 % (95 % CI: 87.9-99.9 %) and specificity of 100 % (95% CI: 91.3-100 %) (Nantavisai et al., 2007).

The molecular detection of parasite DNA begins with the extraction of *Giardia* genomic DNA from stool samples. The principle is: samples are first lysed using proteinase K and buffers are used to provide optimal DNA binding conditions. The lysate is loaded onto the DNeasy Mini spin column and that is followed with centrifugation. During centrifugation, DNA selectively binds to the DNeasy membrane as contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in water or buffer, ready for use (Figure1.5). This is the method adapted

for use on stool samples for this project. For detailed extraction procedure see Section 2 and the DNeasy® Blood & Tissue Handbook, Pg 28-30 (DNeasy Blood and Tissue Handbook, 2006).

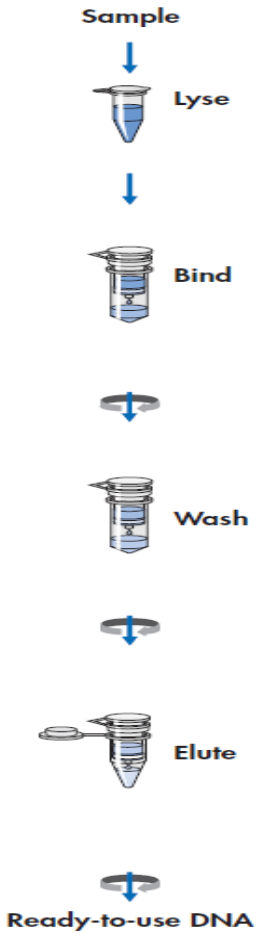


Figure 1.5: DNeasy mini procedure.

With the DNA extracted, the appropriate primers and probe are then deployed to set up the PCR as discussed in Section 2. The primer selection takes into consideration the purpose of the PCR whether it is for genetic characterization or genus identification. This will determine the oligonucleotide type to use (see Table 1.3). Probe-based real-time methodology is used in this project and therefore along with the primer selection is the choice of an appropriate probe (see Section 2). A number of genetic loci for *Giardia intestinalis* targeted by these oligonucleotides are known. Some of these are

gdh, tpi, (SSU) rRNA, and β-Giardin. Studies have shown that gene polymorphism could group isolates into assemblages (Wielinga & Thompson, 2007). In a study to evaluate the discriminatory power of genotypic markers for identifying nucleotide diversity within sub-genotypes of *Giardia intestinalis*, assemblage B, Siripattanapipong et al. (2007) showed that the combination of two loci provides a higher discrimination power for sub-genotypes of *Giardia intestinalis* than using any single locus. The discrimination powers of gdh, tpi, β-Giardin, ef1-α , and SSU-rDNA genes were 100, 100, 96.43, 42.86, and 0 % respectively (Siripattanapipong et al., 2011). They could not, however, compare the results directly because the datasets were from different sources (Siripattanapipong et al., 2011). The less variable and conserved (SSU) rRNA gene was targeted by the Verweij real-time PCR primers in this project for identification of *Giardia intestinalis* to the genus level. The (SSU) rRNA genes of *Giardia intestinalis* and *Giardia muris* are 80 % similar and this corresponds to about 290 different bases over the length of the gene (Rochelle, De Leon, Stewart, & Wolfe, 1997). Verweij et al. (2003, 2004)) designed and used a set of primers on the basis of the known (SSU) rRNA gene sequence for *Giardia intestinalis* (GenBank accession no. M54878) such that a 62-bp fragment within the (SSU) rRNA gene was amplified and detected specifically for *Giardia intestinalis*.

The gdh gene was also targeted in this project using the Primerdesign Ltd. real-time PCR. This gene has been reported to have a 100 % discriminatory power and is capable of both genotyping and sub-typing *Giardia* isolates (Siripattanapipong et al., 2011). These techniques are very useful but they are also susceptible to contamination and therefore working areas for extraction procedures (“dirty” areas) should be separated from cocktail preparation areas (“clean” areas) in order to minimize cross contamination. Non-viable parasite DNA could also be detected and therefore clinical judgement will be needed in

the interpretation of PCR results for treatment purposes (Josephson, Gerba, & Pepper, 1993).

1.4.3.1 Conventional polymerase chain reaction

The conventional PCR requires a DNA template containing the target region and two primers flanking the marked region (Figure 1.6). After amplification, PCR products are separated electrophoretically on an agarose gel according to size. There is a set of known DNA molecular weights (markers) run on each gel as standards to aid in the determination of sizes of amplicons (Figure 1.7).

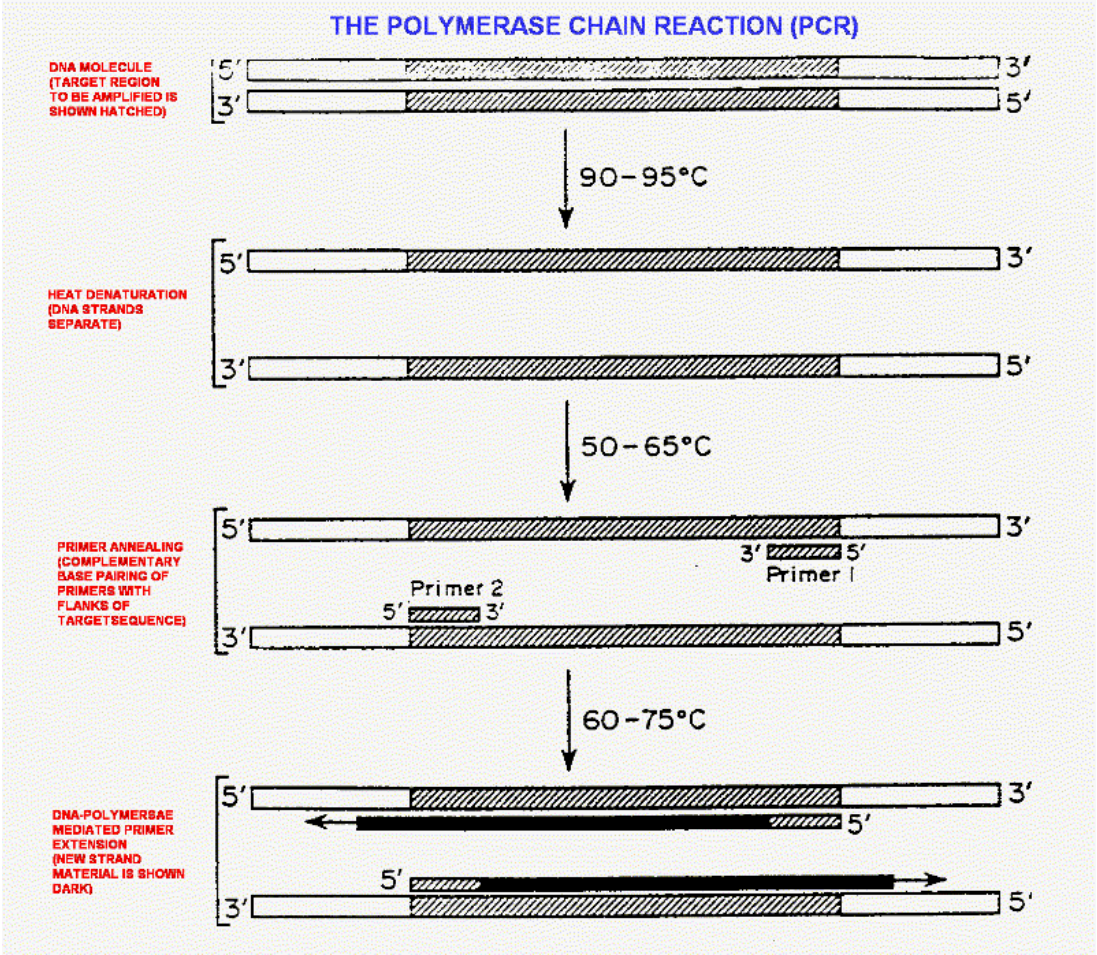


Figure 1.6: Steps in the polymerase chain reaction.
(source: http://users.wmin.ac.uk/~redwayk/lectures/images/PCR-1.gif).

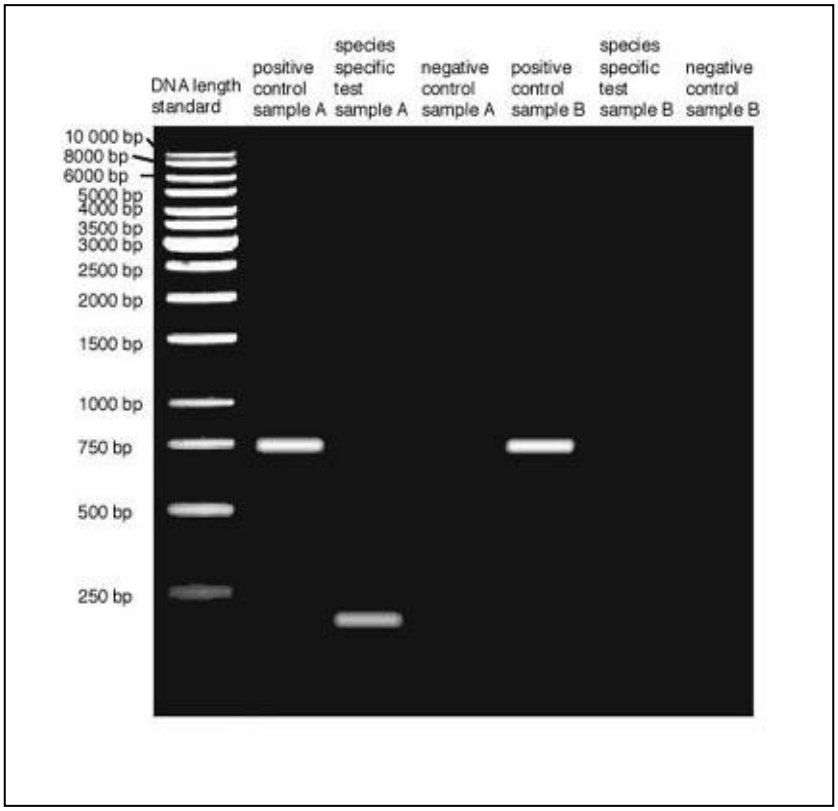


Figure 1.7: Gel electrophoresis image.
The DNA marker is in lane 1 and shows the range of sizes from 250 bp to 10000 bp (Source: soft-matter.seas.harverd.edu).

For this study, the primers, and their sequences, used to amplify a 292-bp region of the 5' end of the (SSU) rRNA gene of *Giardia intestinalis* were RH 11, forward primer (1- 18), 5'CATCCGGTCGATCCTGCC3' and RH 4, reverse primer (268- 292), 5'AGTCGAACCCTGATTCTCCGCCAGG3' (Hopkins et al., 1997).

To further increase the sensitivity and specificity of the amplification procedure, a second round of amplification with a second pair of oligonucleotide primers was used in a nested PCR for increased amplification products for *Giardia* DNA. The designed primers for the second round PCR were:

GiarF: 5'GAC GCT CTC CCC AAG GAC3' and

GiarR: 5'CTG CGT CAC GCT GCT CG3' (Read, Walters, Robertson, & Thompson, 2002).

1.4.3.2 Real-time polymerase chain reaction

In real-time PCR, the amplification product is measured at each

Cycle and the initial quantity of the target can be determined quantitatively. The TaqMan chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR.

The basic steps of the process involve the use of an oligonucleotide probe. The probe contains a reporter fluorescent dye on the 5´ end and a quencher dye on the 3´ end (Figure 1.8). In an intact probe, fluorescence emitted by the reporter dye is quenched by the quencher molecule by fluorescence resonance energy transfer (FRET) through space.

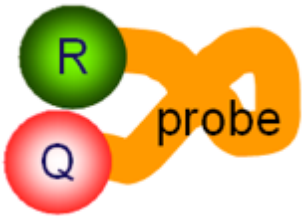


Figure 1.8: Illustration of a TaqMan® probe in solution.
R represents the reporter dye, Q, the quencher molecule, and the orange line, the oligonucleotide. (Source: Life Technologies – Real-time PCR handbook, 2012)

If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5´ nuclease activity of Taq DNA polymerase as this primer is extended (Figure 1.9).
This cleavage of the probe separates the reporter dye from the quencher dye, increasing the reporter dye signal. It also removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. As additional reporter dye molecules are cleaved from their respective probes with

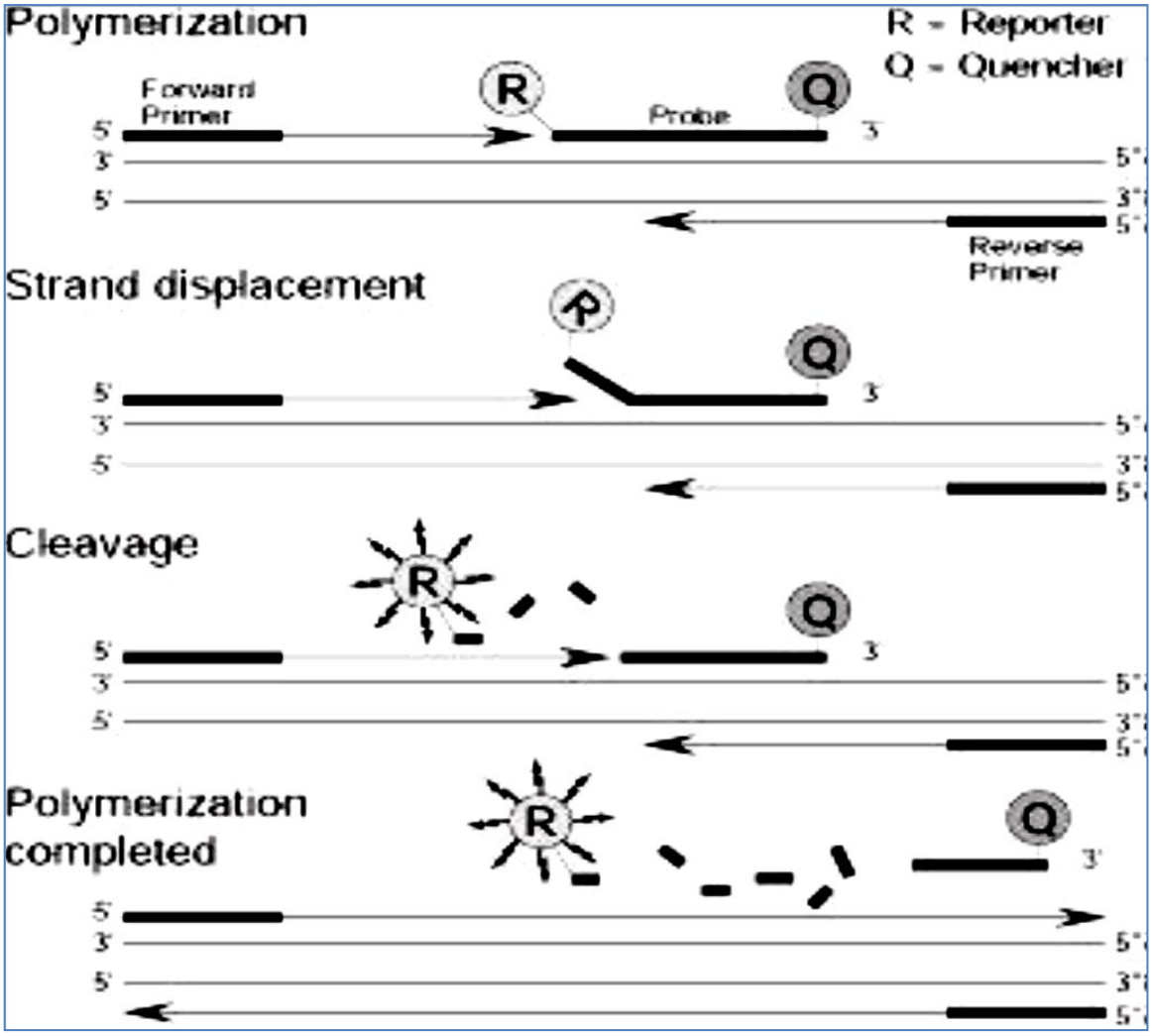


Figure 1.9: TaqMan® probe.
The TaqManR probe has a gene-specific sequence and binds the target between the two PCR primers. Attached to the 5' -end of the TaqManR probe is the "reporter," fluorescent dye. On the 3' -end of the probe is a quencher. The quencher also blocks the 3_end of the probe so that it cannot be extended by thermostable DNA polymerase (Source: Applied Biosystems – Essentials of real time PCR; http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_039996.pdf).

each cycle, the resulting increase in fluorescence intensity is proportional to the amount of amplicon produced.

Real-time PCR provides for a high-throughput analysis in a closed system and considerably reduces the problems of cross-contamination. It is quantitative in as much as the cycle threshold is related to the starting number of the copies of the target fragment. Real-time PCR has been developed for *Giardia* using

TaqMan probe detection chemistry, where increased fluorescence takes place following hydrolysis by Taq DNA polymerase (Verweij et al., 2004; Verweij et al., 2003). This has led to various PCR approaches targeting different genetic loci to differentiate species and assemblages of *Giardia* (Almeida, Pozio, & Cacciò, 2010; Cacciò, De Giacomo, & Pozio, 2002; Read, Monis, & Andrew Thompson, 2004). Chalmers and Katzer (2013) quoting from Taniuchi et al. (2011) reported a PCR approach that replaces probes with carboxylated Luminex beads(R. M. Chalmers & Katzer, 2013).The assay uses two multiplex PCR reactions with specific primers for protozoa and helminths and replaces probes with beads. PCR products attached to beads are detected on a Luminex platform. When compared to the probe based PCR, the multiplex PCR-bead assay produced an improved sensitivity and specificity of 83 % and 100 % respectively (Taniuchi et al., 2011). Multiplexing the assay widens the diagnostic screen for a large panel of intestinal parasites.

1.4.3.3 DNA sequencing

Post-PCR analyses are usually based on the direct sequencing of the amplification products or on the digestion with endonucleases followed by gel-electrophoresis of the restriction fragments. In the last few years, the molecular characterization of a large number of isolates, collected from infected hosts and from the environment, has considerably added to the body of information about the zoonotic potential and the epidemiology of giardiasis. Indeed, two assemblages (A and B) of *Giardia intestinalis* have been established as human pathogens. DNA sequencing has delineated three main groups based on small sub-unit (SSU) rRNA sequencing and tim (triosephosphate isomerase gene; could also be written as “tpi”) sequencing (Baruch, Isaac-Renton, & Adam, 1996; Cacciò, 2004).

1.4.4 Stool culture

Stool culture is not useful for diagnosing giardiasis because the organism cannot readily be grown from patient samples. The lack of a cell culture model for *Giardia* has also made the determination of viability, infectivity, and virulence using molecular methods a technical challenge (Alum, Sbai, Asaad, Rubino, & Khalid Ijaz, 2012). PCR has, however, been successfully combined with the electrophysiological analysis of cell culture (ECC-RT-PCR) post-trophozoite attachment. The researchers have concluded that this new integrated cell culture assay, can be used as a rapid and cost-effective tool for assessing the viability and infectivity of environmental isolates of *Giardia* sp cysts (Alum et al., 2012).

1.5 The search for a new assay at Hospital for Tropical Diseases

It has been observed at the Hospital for Tropical Diseases (HTD) in London that for some patients with chronic diarrhoea and malabsorption, stool microscopy results are repeatedly negative despite ongoing suspicion of giardiasis. P. Chiodini (personal communication, April 11, 2008) commented that when this group of patients is treated empirically for giardiasis using tinidazole many of them get better. In a study by Bolin, 1982, there was a 9 % prevalence rate of giardiasis among 100 consecutive patients with chronic diarrhoea. Fifteen patients without a definitive diagnosis responded to empiric metronidazole or tinidazole therapy (Bolin, Davis, & Duncombe, 1982). In another study, patients initially diagnosed with severe irritable bowel syndrome (IBS) were treated for *Giardia* with metronidazole and all symtoms resolved (Gunasekaran & Hassall, 1992). A patient with biliary tract dysfunction was also treated for giardiasis and all the patient's symptoms disappeared and gall bladder visualization returned to normal (Goldstein, Thornton, & Szydlowski, 1978). A more sensitive diagnostic test could help in explaining these situations.

It is possible the parasite burden, on these occasions, was below the detection level of the test used.

Figure 1.10 shows the lines of approach when there is a clinical suspicion of giardiasis and treatment is contemplated.

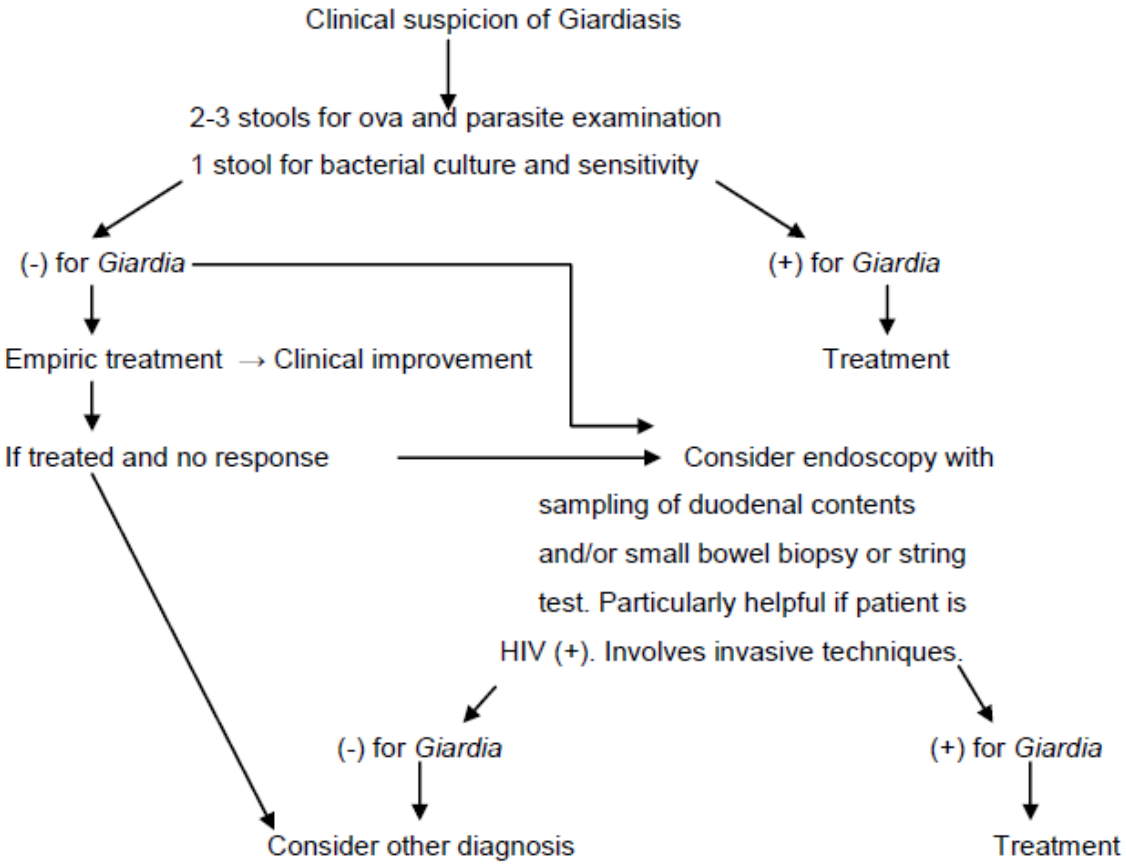


Figure 1.10: Diagnosis and management of suspected giardiasis.
(Source: Modified from Gardner & Hill, 2001).

In the unusual case where a diagnosis cannot be made by looking for the parasite in stool samples, endoscopy with duodenal fluid sampling and biopsy may be performed. In human immunodeficiency virus-infected patients with diarrhoea, whose illness has multiple potential etiologies, this approach will be preferred to empiric treatment by some clinicians (Gardner & Hill, 2001). Invasive methods like the string test and duodenal aspirate examination may be required to detect the trophozoite stage of *Giardia* (Adam, 1991) in cases where

clinical symptoms persist but the stool microscopy results have been consistently negative (Figure 1.10). This is particularly useful in immunocompromsed patients (HIV+) who because of ineffective immune system tend to suffer from gastrointestinal infections that could resemble giardiasis. Tinidazole is prescribed for chronic diarrhoea in the HTD outpatients' department as presumptive therapy for giardiasis. In a small study at HTD to investigate whether empirical therapy with tinidazole could be justified by comparing outcome in those with proven giardiasis and those without, one hundred and thirty six patients were studied and of this group, 23 had proven giardiasis. Fifteen patients of the 23 with proven giardiasis had complete symptom relief (65 %); 113 patients had empirical therapy and 64 of them had complete symptom relief (57 %) (Crane, Whitehorn, & Wright, 2008). The authors concluded that it was possible patients who did not show a clinical improvement may have had another pathological process causing their symptoms. In addition, those who did improve after tinidazole treatment despite having negative stool tests for *Giardia* may have had another infection that responded to therapy with tinidazole or had a *Giardia* infection that was not detected. The outcome of this study has highlighted the need for a more sensitive diagnostic test for the diagnosis of *Giardia* infections. The routine diagnostic test for identifying this parasite in the Department of Clinical Parasitology is ova, cyst and parasite-microscopy (OCP-M). This method uses a combination of three tests to look for *Giardia intestinalis.* The method, however, is not specific for *Giardia intestinalis* as it can detect other parasites if they are present. The three tests are: the formol-ether concentrate for ova, cysts, and parasites (OC&P), wet preparation, and rapid Field's stain. Although considered as the "gold standard", the OC&P test, as a standalone method is time-consuming and highly dependent on the skills of the microscopist. Also the OC&P method detects cysts only (Allen & Ridley, 1970) so additional tests must be performed to detect trophozoites. That is the reason why in the

Department of Clinical Parasitology, wet preparations and smears for staining are also made when the stool is unformed or liquid. Because of these additional tests, the method is called ova, cysts, and parasites with microscopy (OCP-M) to differentiate it from the OC&P which does not include these additional tests. Even with the much improved OCP-M over the standard OC&P method, the problem of microscopy negative clinical giardiasis is still encountered. This emphasises the importance of more sensitive diagnostics (Crane et al., 2008). Perhaps the parasites are in such low numbers that they are below the detection level of the OCP-M method as well.

Even though an attempt has been made to overcome some shortcomings of the OC&P method by using the OCP-M, there still remains the problem of intermittent shedding of cysts which has led to the requirement for patients to submit three consecutive stool samples for examination before they are declared likely to be free from giardiasis (Figure 1.10). A single stool sample will be preferred instead of three.

The problems connected with the laboratory diagnosis of giardiasis as discussed above have resulted in the investigation of a molecular-based approach to diagnosing this gastrointestinal infection. Good sensitivity and specificity levels have been reported in the literature for molecular-based methods for diagnosing giardiasis. However, incorporation of these methods into the routine diagnostic laboratory is time consuming and increases the cost of a stool examination (Haque et al., 2007; Verweij et al., 2004).

The Department of Clinical Parasitology is the diagnostic laboratory for HTD and offers a reference/referral service and therefore it is expected that these diagnostic problems will be addressed in a way that provides the best possible service for patients/users. The undertaking may be expensive but the service provided will be commensurate with that of a reputable reference laboratory offering the best service. The practical benefits of a successful

project upon finding a very sensitive test and adopting it into routine practice will include the following:

1. Patients may no longer be required to submit three stools for the laboratory diagnosis of giardiasis before they can be declared free of the parasite. One sample may be sufficient.
2. Invasive techniques like duodenal biopsy or aspirate may no longer be actively considered for the diagnosis of giardiasis. Asymptomatic giardiasis could be picked up in the investigation of an outbreak or differential diagnosis of other conditions. In one study 6.5 % of patients with irritable bowel syndrome had *Giardia* (Grazioli et al., 2006).
3. Patients may no longer be treated for giardiasis based on clinical suspicion alone (empirical treatment).

1.5.1 Purpose and aim for the research

OC&P remains the *de facto* gold standard for the laboratory diagnosis of intestinal parasites which include *Giardia intestinalis*. A non-microscopy based test would be desirable. Since Mullis won the Nobel Prize in chemistry in 1993 for his invention of the PCR technique, the methodology has reached sufficient technical maturity to be considered for routine use in clinical laboratories using a standardized protocol. Therefore, this study was conducted to determine the of real-time PCR methodology in the diagnosis of giardiasis. An attempt will be made to evaluate some selected non-microscope- based methods used to diagnose *Giardia* infection with a view to finding the best test to diagnose giardiasis. These tests will be compared with the OCP-M method currently in use which requires the use of a microscope to look for the parasite itself. The non-microscope-based tests selected for inclusion in the assessment protocol will be those that detect components (e.g. antigens and DNA) of the parasite rather than the whole intact parasite. The question answered in this study was: In patients with suspected *Giardia intestinalis* infection (giardiasis), would the

use of non-microscopy-based tests, when compared with the light microscopy test (OCP-M), lead to improved detection of the parasite in human stool samples? Consequently, the aim of this study was to determine the sensitivity and specificity of OCP-M and real-time PCR assay for *Giardia intestinalis* detection by using a composite reference standard test of enzyme immunoassay and rapid membrane test.

1.5.2 Objectives of the study

Six objectives for this research are described in Table 1.4.

Table 1.4: Description of objectives.

Objective no.	Description
01	To carry out an exploratory investigation using spiked stool samples to determine any differences in performance between the index tests: Ova, cyst and parasite-microscopy (OCP-M) and real-time PCR.
02	To carry out an exploratory investigation using spiked stool samples to ascertain the analytical sensitivity and specificity of the reference tests: Enzyme immunoassay (EIA) and rapid membrane test (RMT)
03	To evaluate and compare the accuracy measures for OCP-M and real-time PCR using a non-probability sampling technique with consecutive samples.
04	To compare the accuracy measures for real-time PCR to that of a combination of OCP-M and real-time PCR.
05	To investigate the effect of different storage conditions on the detection of *Giardia intestinalis* DNA in human stool samples.
06	To develop an algorithm and a business plan for use in the Department of Clinical Parasitology for the laboratory investigation of giardiasis incorporating real-time PCR.

1.5.3 Ethics

Before this project started, an application for the assessment of it for ethical review was submitted to the NHS Research Ethics Committee (NHS REC). After considering it in the light of how they differentiate research from other activities, it was decided that this project did not require ethical review (SL24 Project version 4.0 ref. 09L 446). Upon their advice, I checked with the local R&D Department for any further review arrangements or sources of advice that may apply to projects of this type. Again, the reply received from the senior research administrator for the joint UCLH/ UCL Biomedical research unit at University College London Hospitals NHS Foundation Trust (UCLH) indicated that no further review was required since the study was considered as an audit (P. Diamond, personal communication, January 04, 2010). With these decisions received, the project then began.

Chapter 2: Analytical verification of diagnostic tests for *Giardia intestinalis*

2.1 Introduction

The requirement for verification and validation procedures for assays in general is discussed in the international quality standard for medical laboratories ISO 15189:2007 (Raymaekers, Smets, Maes, & Cartuyvels, 2009). The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) document ICH Topic Q 2 (R1) published by European Medicines Agency also provides guidance on validation of analytical procedures (Procedures, 1995). Another document that also provides information on validation and quality control of the PCR methods used for the diagnosis of infectious diseases is the Office International des Epizooties (OIE) Terrestrial Manual 2008 ("OIE Terrestrial Manual," 2008), published by European Medicines Agency. With the proliferation of both commercial and non-commercial assays for the detection of *Giardia*, the need for standardization and quality assurance are required. Sloan (2007) quoting from the Clinical and Laboratory Standards Institute document CLSI MM3-A2, defined verification as the one-time process completed to determine or confirm test performance characteristics before the test system is used for patient testing, and validation as the continuous process of proving that a procedure, process, system, equipment, or method works as expected and achieves the intended results. Components of validation are quality control, proficiency testing, employee competence, and instrument calibration (Sloan, 2007). Presently, the

challenges to the clinical microbiology laboratory for introducing assays like the real-time PCR test systems include what verification experiments are required and how many and which types of specimens should be tested (Sloan, 2007). For the purpose of this study, publications available from agencies ICH, OIE, and CLSI together with the publications of Raymaekers et al. (2009) and Sloan (2007) assisted in providing guidelines for establishing and confirming the performance characteristics and accuracy measures of the diagnostic tests evaluated in this research project. For analytical verification, the three main areas chosen for investigations were analytical sensitivity, analytical specificity, and PCR reaction efficiency.

Analytical sensitivity, as opposed to diagnostic sensitivity, is the lowest detection level of a test (Saah & Hoover, 1997). It is also known as limit of detection (LOD). Analytical specificity, on the other hand, refers to the ability of the test to detect, e.g. *Giardia intestinalis*, rather than any other parasite in the stool sample.

Analytical specificity was investigated for the five tests by using a pooled *Giardia*-negative stool with various types of parasitic, bacterial, and fungal organisms: *Entamoeba coli*, *Endolimax* nana, *Entamoeba histolytica/dispar*, Yeasts, *Escherichia coli*, *Klebsiella sp*., *Citrobacter sp*., *Proteus sp*., *Enterococcus faecalis*, *Cyclospora cayetanensis* and *Cryptosporidium sp*. Bacterial pathogens were not available but have already been tested in a previous study (Verweij et al., 2003). The pooled *Giardia*-negative stool and the serially diluted samples for the trophozoites and cysts were all tested with the two real-time PCRs, OCP-M, EIA, and the RMT.

Real-time PCR, also called quantitative PCR or qPCR, provides the amount of a target sequence or gene that is present in a sample. The efficiency of a PCR reaction has effect on the threshold cycle (Ct.), where Ct is the intersection between an amplification curve and a threshold line (Figure 2.1). It is a relative measure of the concentration of target in the PCR reaction (Real-

time PCR: Understanding Ct, 2011). A positive signal in real-time PCR is detected by the accumulation of fluorescence. The Ct is inversely proportional to the amount of starting nucleic acid target in the sample. In order words, the lower the Ct level the greater the amount of target nucleic acid in the sample and vice versa.

Figure 2.1: Interpretation of amplification curves
(source: http://www.wvdl.wisc.edu/PDF/WVDL.Info.PCR_Ct_Values.pdf)

The actual numerical values of the Cts depend on the reagents and the real-time thermocycler used and may not be used as the basis for comparing two Real-time PCR methods. The following observations have been made and are generally accepted: Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample. Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid. Cts of 38-40 are weak reactions

indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination(Real Time PCR Ct Values, n.d.).

Efficiency reflects the performance of the assay in achieving what it has been designed for. It is derived from the function for the amount of PCR product formed: $N = N_0 \times E^n$. N represents the number of amplified molecules and N_0 is the initial number of molecules. The number of amplification cycles is n and E is the efficiency which is ideally 2. The standard curves are derived from the function described above: $n = -(1/\log E) \times \log N_0 + (\log N/\log E)$. Therefore, the slope of the line equals -(1/ log E) and the efficiency can be calculated from this (Mygind et al., 2002).

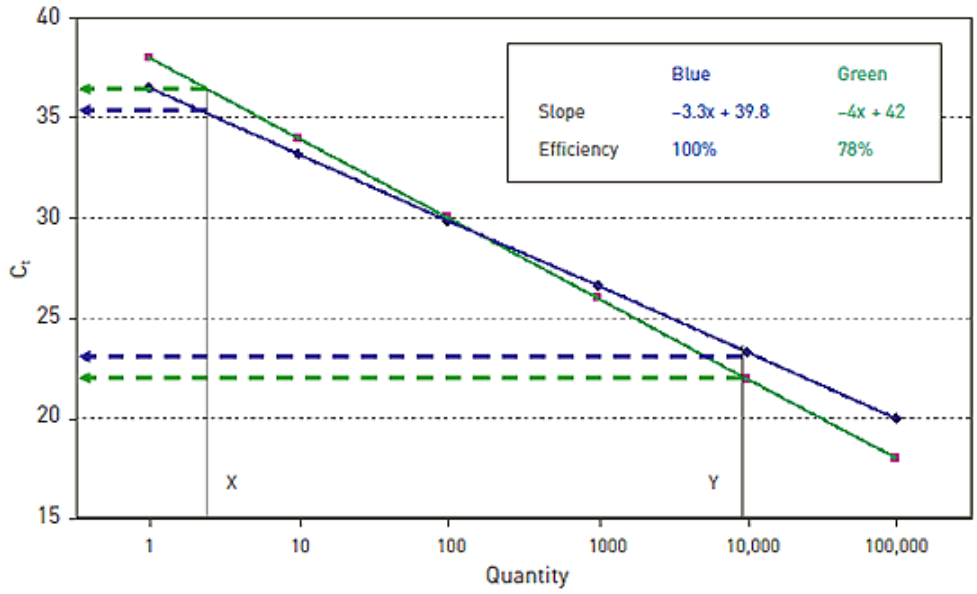


Figure 2.2: Comparison of standard curves.
The blue curve has an efficiency of 100 % (the slope is –3.3). The green curve has an efficiency of 78 % (the slope is –4). Amplification of quantity Y gives an earlier Ct under low efficiency conditions (green) compared to the high efficiency condition (blue). With a lower quantity (X) there is an inversion and the low efficiency condition (green) gives a later Ct than the high efficiency condition (blue) (Real-time PCR: Understanding Ct, 2011)

Low efficiency conditions produce a standard curve with a different slope from that under high efficiency. In Figure 2.2, two samples (X and Y) amplified under low and high efficiency conditions show different Ct values for the same

target concentration. The high-efficiency condition (the blue curve) gives a later Ct at high concentrations and a better sensitivity at low target concentrations.

The PCR efficiency is dependent on the assay, the master mix performance, and sample quality. Efficiency between 90 and 110 % is generally considered acceptable. The nearer the value to 100 %, the better the efficiency (Real-time PCR: Understanding Ct, 2011). Reaction efficiencies were compared between the two named real-time PCRs.

This early phase of the project was designed to verify the analytical potential of all the tests (i.e. both index and reference tests) deployed in this study. In order to achieve this, *Giardia* positive stool, obtained from routine practice, was liquefied with PBS pH 7.2 and *Giardia* cysts were counted with the aid of C-Chip counting chamber following the manufacturer's guidelines (Appendix I). One in five serial dilutions were prepared in a *Giardia*-negative stool which was also liquefied with PBS pH 7.2 to give the following range of dilutions with estimated cysts numbers/ml (Table 2.1).

Table 2.1: Serial dilutions of a *Giardia positive* stool sample.
***Giardia* positive stool sample was serially diluted one in five and used for the estimation of detection limits of the diagnostic tests. Cyst numbers in tube 1 were counted with C-Chip counting chamber and cysts numbers in tubes 2 to 7 were estimations based on the serial dilutions.**

Sample tube no.	1	2	3	4	5	6	7
No. of cysts/ml	71, 000	14,200	2840	568	113.6	22.7	4.5

Giardia intestinalis trophozoites (Alexeieff Portland-1 strain) were also similarly counted using C-Chip counting chamber and 1 in 10 serial dilutions prepared for the estimation of detection limit (Table 2.2).

Table 2.2: One in ten serial dilutions of *Giardia intestinalis* culture.
An axenic culture of *Giardia intestinalis* (Alexeieff Portland-1 strain), was used in LOD testing. *Giardia* trophozoites in tube 1 were counted with C-Chip counting chamber and trophozoites numbers in tubes 2 to 7 were estimated following the serial dilutions. The dilutions were prepared in *Giardia*-negative stool.

Sample tube no.	1	2	3	4	5	6	7
No. of trophozoites/ml	$9.2x10^4$	$9.2x10^3$	$9.2x10^2$	92	9.2	0.92	0.092

The cysts and the trophozoites dilutions were tested with all the five diagnostic assays deployed in this study: OCP-M, RMT, EIA, Primerdesign Ltd. real-time PCR, and Verweij real-time PCR. The methods for testing have been discussed under Section 2.2.

In order to standardize and compare the amplification efficiencies of the Verweij real-time PCR and the Primerdesign Ltd. real-time PCR, 10-fold dilutions were prepared from the DNA solution in Tube 1 of Table 2.1 after diluting it 1 in 2 first and then followed by the 1 in 10 dilutions (Table 2.3). The DNA was extracted from a stool sample with a known number of *Giardia intestinalis* cysts (7.1×10^4 cyst/ml of stool) as determined by light microscopy.

Table 2.3: One in 10 serial dilutions of *Giardia* DNA extracted from a *Giardia* positive stool.
***Giardia* positive stool sample was serially diluted 1 in 10 and used for the standard curve construction. Cysts in Table 2.1 tube 1 were counted with C-Chip counting chamber to obtain a value of 7.1×10^4 cysts/ml of stool. The DNA extracted from this stool was then diluted 1 in 2 with nuclease-free water to give the estimated value of 3.55×10^4 cysts/ml. It was this latter value that was serially diluted 1 in 10 to provide the serial dilutions for the standard curve.**

Sample tube no.	1	2	3	4	5
No. of cysts/ml	35,500	3,550	355	35.5	3.55

The range of dilutions for the standard curve was chosen to offer the maximum chance of detecting *Giardia* DNA in every dilution tube by both real-time PCRs. As the dilutions were for efficiency determination and not LOD per

se, the lower end concentrations were, therefore, not extended to 1 or 2 cysts/ml. Only the two real-time PCRs (Verweij et al. and Primerdesign Ltd.) were used to test these 1 in 10 serially diluted *Giardia* DNA solutions.

2.2 Methods

The reference test was a composite of EIA and RMT with a conventional PCR (CPCR) used only to resolve discrepant test results. And the index tests were ova, OCP-M, Verweij real-time PCR, and Primerdesign Ltd. real-time PCR.

2.2.1 Ova, cyst, and parasite microscopy

This is a formol-ether sedimentation technique for concentrating parasites in stool samples for identification microscopically. The method is not directed at *Giardia intestinalis* alone, it can detect other parasites as well. It maximizes the numbers of organisms detected when they are too scanty to be seen otherwise (Allen & Ridley, 1970; Ridley, 1956). The examination of stained direct faecal smears for vegetative forms of parasites was not part of the methodology initially. The procedure has been referred to as "ova, cyst and parasite" (OC&P). It was later on that modifications to the OC&P emerged. A modified form of the OC&P is used at HTD-Clinical Parasitology and this will be described fully in this section and evaluated in this project. Ridley and Allen faecal concentration technique is non-invasive and detects mainly the cyst form of *Giardia intestinalis* in stool samples. Its usage as a diagnostic technique has, however, the problem of compliance. Because three negative stool samples, taken on separate days, are required for the confirmation of cure after treatment or for the confirmation of a negative diagnosis following clinical suspicion of giardiasis, patients are less inclined to comply.

The method uses the Parasep faecal parasite concentrator (Figure 2.3). Three 3 ml of either ethyl ether or ethyl acetate was used to remove fat and

debris from 1 g (1 ml if liquid) of stool with 6 ml of 10 % formalin added to kill bacteria and other microbial flora in a mixing chamber (Figure 2.3). After filtration, followed by centrifugation at 3000 rpm for 1 min, the parasite was left in the pellet at the bottom of the sedimentation cone with faecal debris trapped in the catchment area above the filter (Figure 2.3). Fat in the form of a plug was left in the ether layer on top of the formalin column.

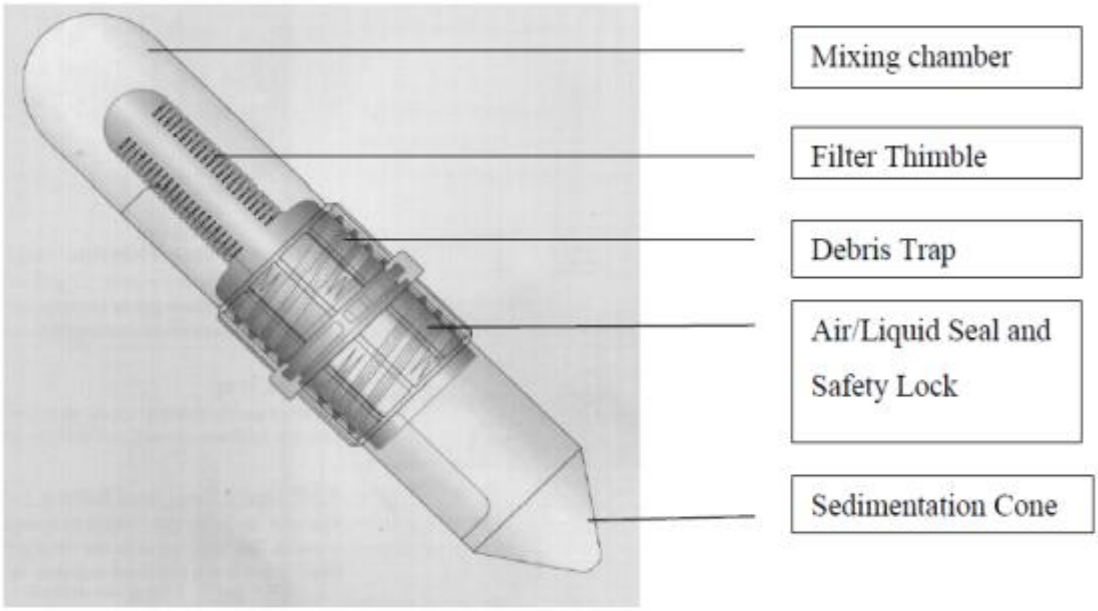


Figure 2.3: Parasep faecal parasite concentrator.
(Image source: DiaSys Ltd. UK, http://www.diasys.com/products/parasitology/midi-parasep/)

The filter had 425 µm pores that allowed parasites to go through and larger faecal debris held behind it. After discarding the formalin-ether layer, a couple of drops of normal saline were used to reconstitute the pellet which was then examined microscopically. A drop of iodine was used to identify internal structures of cysts.

When unformed or liquid stools were encountered, direct wet preparations and rapid Field's stained faecal smears were also prepared to supplement the spun deposit to look for trophozoites of *Giardia intestinalis.* In this project, this approach has been referred to as OCP-M to differentiate it from the original Allen and Ridley OC&P test. All the 213 samples that were

archived for this study were processed in the course of normal routine practice using Parasep. They were examined microscopically with the addition of wet preparations and stained smears on all unformed and liquid samples.

2.2.2 Rapid membrane test

Giardia-Strip

This is a single one-step immunochromatographic membrane test. The kit is called *Giardia*-Strip and Coris BioConcept is the manufacturer of this commercial diagnostic kit. The principle behind this ready-to-use test is: monoclonal antibodies raised against *Giardia intestinalis* cyst's membrane antigens are conjugated with colloidal gold and the conjugate is deposited or localized on a polyester membrane. When the strip is dipped into a diluted faecal sample, the solubilised conjugate migrates with the sample by passive diffusion and any *Giardia* cysts that might be present forms a complex with the conjugate and the complex becomes immobilized when bound to the anti-*Giardia* reagent. This results in the development of a red line at the specified location on the strip. Any unbound conjugate continues to migrate and then comes into contact with a second reagent line, an anti-chicken IgY polyclonal antibody. The resulting red line confirms that the test is working properly. The test result is visible within 15 minutes.

Procedure (Figure 2.4):

1. Using a pastette, 0.5 ml (15 drops) of the dilution buffer solution were put in a 3 ml tube
2. Using a 10 µl loop, 2 loops of liquid stool (1 loopful when the stool was formed) were added to each tube from 1. The loop was used initially to break up formed stools before mixing the sample with them.

The tubes were vortexed to homogenize each sample preparation.

Using plastic disposable pipettes the contents of all tubes were transferred into a corresponding deep-welled micro titre tray, with appropriately labelled

positions. The sampling loop and pastettes were discarded and appropriately labelled sensitized strips were applied to each well in the direction indicated by a red arrow (Figure 2.4). The set up was left at room temperature for 15 minutes. Positive test results were observed earlier when both the control line and the test line became visible.

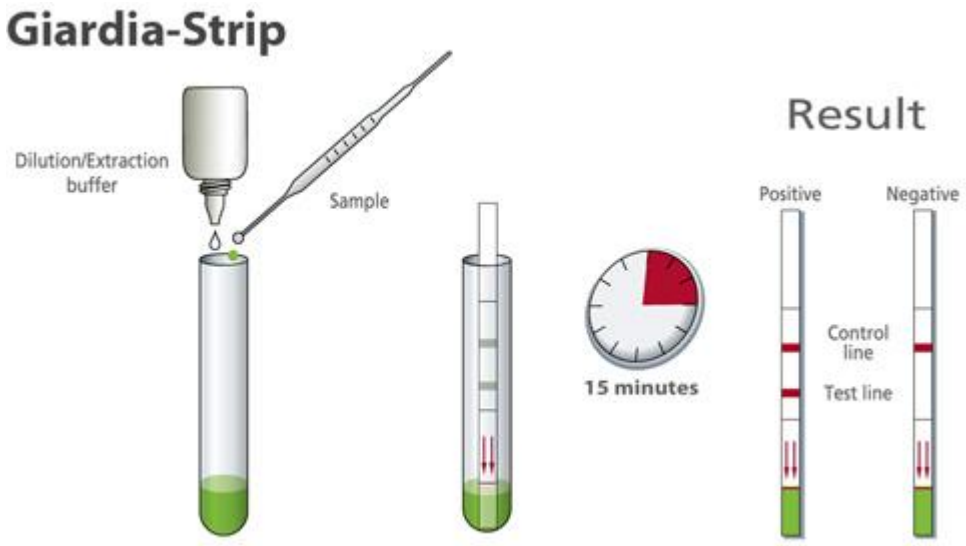


Figure 2.4: *Giardia*-strip procedure.
(Courtesy from manufacturer's website: http://www.corisbio.com/Products/Human-Field/*Giardia*.php)

Interpretation of results

The results were read within 15 minutes following the manufacturer's instructions.

Negative test result: only one (1) red line (upper). No other band was present

Positive test result: two (2) red lines. Any signal on the test line was recorded as a positive test

Invalid test result: No detectable reddish-purple band at the control line (upper)

A couple of the tests failed and had to be repeated because of improper diffusion of the faecal suspension thought to be due to the high viscosity of the samples. Vortexing the samples for longer rectified the situation.

The *Giardia* strip by Coris BioConcept was chosen for this study because it detected *Giardia* cysts membrane bound antigens with the manufacturer's quoted excellent figures for sensitivity and specificity, using microscopy as the gold standard, as 96.3% and 97.8% respectively. It was quick and easy to set up and results were obtained after 15 minutes of waiting. The company is also based in the UK and this offered convenience when dealing with the manufacturer regarding purchases and deliveries. The cost of the strips was also affordable at £55 per a kit capable of performing 25 tests.

See Appendix II for further information regarding the use of this kit.

2.2.3 Enzyme immunoassay (EIA)

Giardia II (TECHLAB) Kit

The *GIARDIA* II test is an enzyme immunoassay used for the qualitative detection of *Giardia intestinalis* cysts antigen in human stool samples. The test uses monoclonal and polyclonal antibodies to detect soluble cell-surface antigen of *Giardia intestinalis*. This diagnostic kit is developed and manufactured by TECHLAB. The principle of test is as follows:

A microassay plate is coated with monoclonal antibody to *Giardia intestinalis* cell-surface antigen. The immobilized monoclonal antibodies bind any *Giardia intestinalis* antigen if present in the stool. Upon the addition of conjugate, a complex is formed which is composed of the monoclonal antibody, the *Giardia intestinalis* antigen, and the conjugate. After washing the plate to remove any unbound materials, substrate is added and the enzyme portion of the conjugate in this enzyme-antibody-antigen complex breaks down the substrate with the development of colour that may be estimated visually or read calorimetrically at 450 nm wavelength (Figure 2.5)

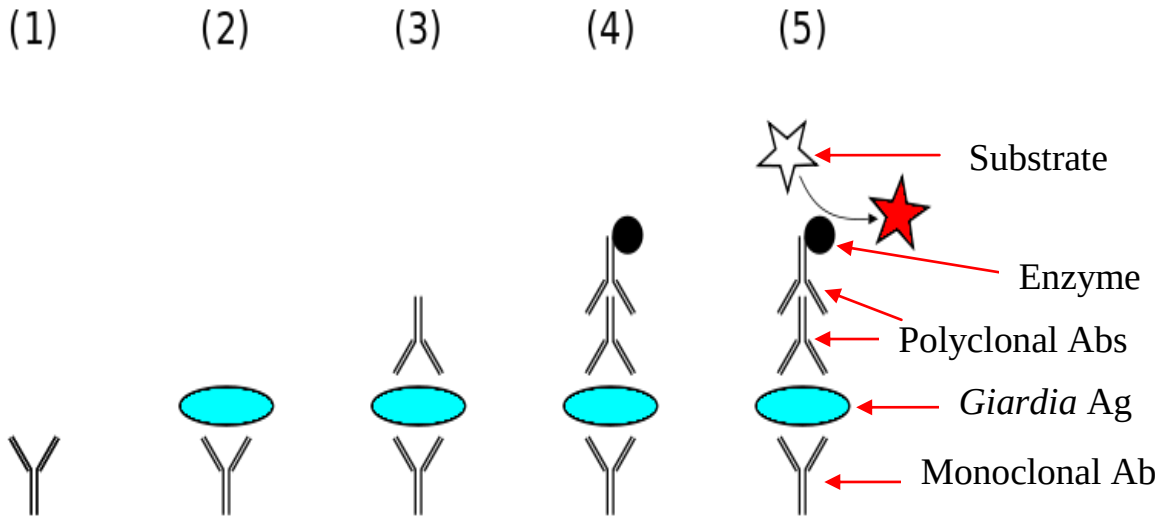


Figure 2.5: The principle of sandwich EIA.
Polyclonal antibodies – Enzyme complex constitutes the conjugate that leads to the breakdown of the substrate with the development of colour after the conjugate has attached to the monoclonal-antigen complex.

Method:

1. One in five dilution was prepared for each test sample (samples were previously stored at -20 ^{o}C with no preservative added) by adding 100 µl of liquid stool (0.1 g if formed) to 400 µl of diluent.
2. One hundred µl of diluent was transferred into each test well of the microassay plate.
3. One drop (50 µl) each of diluted samples from 1 was added to corresponding test wells of the micro-assay plate containing the 100 µl diluent.
4. The micro-assay plate was gently tapped on the sides to mix and left covered at room temperature for 1 h. The contents of the assay wells were tipped into a discard jar and the assay plate washed for a total of 4 times using the x1 wash solution, striking the plate on a paper towel in between washes to remove residual liquid.

5. One drop (50 µl) of conjugate (red cap) was added to each well and gently tapped to mix. The plate was covered and left at room temperature to incubate for 30 minutes.

6. The washing procedure (Step 4) was repeated.

7. Two drops (100 µl) of substrate (blue cap) were added to each well and the plates gently tapped on the sides to mix. It was then incubated at room temperature for 10 minutes.

8. One drop (50 µl) of stop solution (yellow cap) was added to each well at the end of the 10 minutes incubation. The plate was gently tapped to mix and left at room temperature again for a further 2 minutes for colour development.

10. The addition of the stop solution converted the blue colour to a yellow colour (Figure 2.6), which was read by measuring the absorbance at 450 nm on a Dynamic Microplate Microtek Ds spectrophotometer. The instrument was

Figure 2.6: An EIA plate of an actual test run performed in the course of this project. Positive (yellow) and negative (colourless) controls: First well of top row and first well of second row respectively. Ninety four test samples were assayed and the numbering follow on straight after the negative control running from top to bottom: 1-6, 7-14, 15-22, 23-30, 31-38, 39-46, 47-54, 55-62, 63-70, 71-78, 79-86, 87-94.

blanked against air. Visual recordings were also made.

The kit controls, positive and negative, were included in each run with each series of test specimens and the interpretation of the test results were also made with due reference to Table 2.4. Optical density of < 0.15 at 450 nm was read as negative and ≥ 0.15 was read as positive.

Table 2.4: Interpretation of EIA result.

Absorbance at 450 nm	Visual colour	Interpretation
< 0.15	Clear to slight yellow resembled the negative control well in intensity of colour.	Negative - below detectable limits of assay
≥ 0.15	Pale yellow to strong yellow. Obviously more yellow than the negative control well.	Positive - specimen contains *Giardia* antigen

The *Giardia* II (TECHLAB) kit was chosen for this study because it detected soluble *Giardia* cysts antigens, as opposed to membrane bound antigens as mentioned above for the *Giardia* strip. The manufacturer's quoted excellent figures for sensitivity and specificity, using microscopy as the gold standard, were 100 % and 100 % respectively. The company is based in the UK and this offered convenience when dealing with the manufacturer regarding purchases and deliveries. The cost of the EIA kit was also affordable at £199.34 and £330.88 for evaluation and general pricing respectively. Each kit performed 96 tests. For further information regarding the use of this kit, see Appendix III.

2.2.4 Primerdesign Ltd. real-time PCR

The Primerdesign Ltd. genesig kit for *Giardia* is designed for the *in vitro* quantitation of *Giardia intestinalis* genomes by targeting the glutamate dehydrogenase gene (gdh). The primers and probes specifically detect *Giardia intestinalis* assemblages A and B, the sub-types known to infect humans (Genesig advanced kit handbook, HB10.03.03). This assay is marketed as a

research kit and there does not appear to be any record of it having been assessed independently in a clinical laboratory setting before. The real-time PCR instrument Rotorgene Q 6000 (Corbett Research UK Ltd.), the DNA extraction procedure, and the environmental mastermix used were all available in house and were not exclusively designed for the sole accompaniment of the Primerdesign Ltd. kit. The kit contents relevant to this study and the recommended reagent volume per reaction were: *Giardia intestinalis* specific primer/probe mix (colour code brown for 150 reactions, FAM labelled, BHQ quenched), 1µl; *Giardia intestinalis* positive control template (colour code red for standard curve); 1 in 10 dilutions from 2 x 10^5 copies/µl down to 2 copies/µl to provide solutions for the standard curve; Internal/ extraction control DNA (colour code blue for 150 reactions), 4 µl added before extraction, this recommended volume will not deplete the oligonucleotides in the mastermix at the expense of *Giardia intestinalis* positive patient and control samples.
The Primerdesign Ltd.real-time PCR kit was chosen for this study because the target for detection was the gdh gene of *Giardia intestinalis*, as opposed to the (SSU) rRNA gene, the target for the Verweij real-time PCR. The company is based in the UK and this offered convenience when dealing with the manufacturer regarding purchases and deliveries. The cost of the kit was also affordable at £395 for a kit capable of performing 150 tests. For further information regarding the use of this kit, see Appendix IV.

<u>DNA extraction</u>

This involved three stages:

1. Washing the stools samples
2. Lysis of the *Giardia* parasite, and
3. Extraction of the DNA.

1. Washing the stool samples

This washing step was devised at HTD-Parasitology to remove soluble PCR inhibitors before DNA release and extraction. Stool samples previously stored at 4 ^{o}C were used.

a. About 0.5 g (a pea size) of formed stool or 500 µl of liquid stool (vortexed to mix) was transferred into a 2 ml screw capped sarstedt tube using a wooden applicator.
b. One ml of PBS pH 7.2 was added. The mixture was vortexed to mix well and then spun at 14,000 rpm for 5 min.
c. After discarding the supernatant into a formalin discard container, the pellet was weighed and the weight recorded.
d. Stool samples weighing less than 500 mg had the equivalent of 200 µl of PBS pH 7.2 per 100 mg of stool added to it as shown in the chart given below Table 2.5. This created a 1:2 diluted stock. All suspensions were initially mixed with wooden applicators.

Table 2.5: Guide for creating 1:2 dilutions of stool samples.

Weight of stool (mg)	Amount of PBS (µl)
0-125	250
125-190	375
191-250	500
251-310	625
311-375	750
376-440	875
441-500	1000

When a stool sample weighed more than 500 mg, a 1:1 dilution was created first (Table 2.6) before being diluted subsequently to 1:2 with the PBS (250 µl of 1:1 suspension plus 250 µl of PBS).

Table 2.6: Guide for creating a 1:1 dilution.

Weight of stool (mg)	Amount of PBS (µl)
501-625	625
626-750	750
751-875	875
876-1000	1000

The 1:2 diluted stocks were vortexed to mix until they were homogenized then 200 µl was transferred into a new and labelled 1.5 ml snap top centrifuge tube containing 1 ml of PBS pH 7.2. The samples were vortexed briefly to mix and then spun at 14000 rpm for 5 min. After discarding the supernatant, the pellets were stored at 4 ^{o}C when extraction could not be done immediately.

2. Lyses

DNA was extracted from 0.5 g of formed stool or 500 µl of liquid stool using a modified QIAamp DNA Mini kit protocol for tissue extraction (Qiagen Ltd.) (Polley et al., 2011). Prior to extraction, this was supplemented by the addition of a standardized quantity of *Escherichia coli* transformed with a green fluorescent protein (GFP) gene (Murphy, McLauchlin, Ohai, & Grant, 2007). The GFP served as the extraction control as well as the internal control for the PCR. The following steps were followed:

a. ATL buffer (390 µl) was added to the resultant pellet.
b. Proteinase K (40 µl) was added and the pellet was dislodged and broken up with the pipette tip.
c. Four µl of DNA extraction control (Primerdesign Ltd. kit) and 5 µl green fluorescent protein (GFP) (DNA extraction control for the Verweij PCR) were added to the tube.
d. The mixture was vortexed for 1 minute and incubated at 56 ^{o}C in the heating block for 30 min.

e. After the 30 min incubation, the tubes were vortexed again and then incubated at 56 ^{o}C overnight.

3. Extraction of DNA (Using QIACUBE and Qiagen spin-column kits)

a. After the overnight incubation, the lysed samples were each vortexed for 1 min.
b. Samples were centrifuged at 6500g for 1 min in a micro-centrifuge.
c. Two hundred and forty µl of the supernatant was transferred into safe lock 2 ml tubes (labelled Qiagen sample tubes, RB (2 ml)).
d. Two ml safe lock tubes containing sample supernatants were loaded into the sample block of the Qiacube and then the block was transferred into the Qiacube as per extraction sheet.
e. The QIACUBE was used to extract the DNA using a modification of the tissue program eluting DNA into 100 µl of buffer.

Preparation of standard curve solutions using the positive control template (RED):

1. Nine hundred µl RNAse/DNAse free water was pipetted into 5 tubes and labelled 2-6.
2. One hundred µl of positive control template (RED) was pipetted into tube 2.
3. The tube was vortexed thoroughly.
4. The pipette tip was changed and 100 µl pipetted from tube 2 into tube 3.
5. The tube was vortex thoroughly to mix. One hundred µl was pipetted from tube 3 into tube 4.
6. The serial dilution was continued up to and included tube 6 (Table 2.7).

Table 2.7: Standard curve dilution series.

Standard Curve	Copy Number
Tube 1 Positive control (Red)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

The reaction mix was prepared according to (Table 2.8).

Table 2.8: *Giardia intestinalis* reaction mix (cocktail) per test.
Each item was multiplied by the total number of tests including controls to get the required volume of cocktail for the PCR run.

Component	Volume
Environmental mastermix	10 µl
G.intestinalis Primer/probe mix (BROWN)	1 µl
Internal extraction control primer/probe mix (BROWN)	1 µl
RNAse/DNAse free water (WHITE)	3 µl
Final Volume	15 µl

Five µl DNA extract was added to the final volume of 15 µl to provide a total reaction volume of 20 µl. The following amplification cycles were used: Enzyme activation at 95 ^{o}C for 5 min, followed by 50 cycles of a two-step reaction of 10 s denaturation at 95 ^{o}C and 60 s data collection at 60 ^{o}C. Further information is provided in the manufacturer's Genesig Advanced kit handbook, HB10.03.03 (Appendix IV).

Two replicate known patient-positive controls were run at the same time with the kit control using environmental master mix.

Table 2.9: Interpretation of Ct values.
Ct values of 31±3 are within the normal range. In samples with high *Giardia intestinalis* genome copy number, the internal control may not produce amplification plot. This however does not invalidate the test. It should be interpreted as a positive result.

Within tube signal		Run control		Interpretation
Target	Internal control	Negative	Positive	
+ve	+ve	-ve	+ve	+ve
+ve	-ve	-ve	+ve	+ve
-ve	+ve	-ve	+ve	-ve
-ve	-ve	-ve	-ve	PCR run fail
+ve	+ve	+ve	+ve	PCR run fail

+ve = positive, -ve = negative

The real-time PCR was performed in triplicate on both the serially diluted *Giardia*-positive patient sample and the *Giardia* trophozoites culture to determine the detection level of the assay. Fluorogenic data for *Giardia* DNA was collected through the cycling A. Green channel and the extraction/internal control was detected through the cycling A. Yellow channel. The manufacturer's guidelines were followed in the interpretation of results (Table 2.9). The internal control of a sample with a high genome copy number of *Giardia intestinalis* may fail to produce amplification plot when the mastermix oligonucleotides get used up by the excess *Giardia* DNA. This does not invalidate the test and should be interpreted as a positive experimental result. The positive and negative controls however should produce the expected results otherwise the assay has failed. For further information, see Quantitation of *Giardia intestinalis* genomes see Genesig advanced kit handbook, HB10.03.03 (Appendix IV).

Comparison of environmental mastermix with Precision x2 mastermix

Precision x 2 mastermix supplied by Primerdesign Ltd. for use with the Primerdesign Ltd. kit was compared with environmental mastermix using 36 samples that were tested positive when environmental mastermix with the Primerdesign Ltd. kit and also with the Verweij real-time PCR primers. Only

positive samples were tested because from the LOD data in this study, reactions using environmental mastermix with the Verweij real-time PCR primers produced the lowest detection limit and therefore that became a benchmark for the Precision x 2 mastermix using the primers that came with the Primerdesign Ltd. kit.

2.2.5 Verweij real-time PCR

This real-time PCR, unlike the previous one described above, used the (SSU) rRNA gene target for the identification of *Giardia intestinalis*. It is based largely on the published work of Verweij et al (2004) and has been used extensively in other studies (Calderaro et al., 2010; Haque et al., 2007; Schuurman, van Zwet, Lankamp, van Belkum, & Kooistra-Smid, 2007; Verweij et al., 2004). The Verweij et al PCR has been optimised for use in the Department of Clinical Parasitology and as a fully accredited laboratory with CPA (UK) ltd., the assay has to be validated prior to clinical use. It has therefore been included in this study.

Table 2.10: PCR primers and probes for *Giardia intestinalis*.

Primers and probes	**Oligonucleotide sequence 5'– 3'**
Real-time PCR	
Giardia intestinalis	
Giardia -80F	5'-GACGGCTCAGGACAACGGTT-3'
Giardia -127R	5'-TTGCCAGCGGTGTCCG-3'
Giardia -105T	5'CY5-CCCGCGGCGGTCCCTGCTAG-3'BHQ2
Green fluorescent protein (GFP)	
gpfF	5'-CCT-GTC-CTT-TTA-CCA-GAC-AAC-CA-3'
gpfR	5'-GGT-CTC-TCT-TTT-CGT-TGG-GAT-CT-3'
2gpfROX	5'ROX via Amine C6-TAC-CTG-TCC-ACA-CAA-TCT-GCC-CTT-TCG-3'BHQ-2
Conventional PCR	
RH 11	5'-CATCCGGTCGATCCTGCC-3'
RH 4,	5'-AGTCGAACCCTGATTCTCCGCCAGG-3'
GiarF:	5'-GAC GCT CTC CCC AAG GAC-3'
GiarR:	5'-CTG CGT CAC GCT GCT CG-3'

These primers and probe set consisted of forward primer *Giardia*-80F and reverse primer *Giardia*-127R, and the *Giardia intestinalis*-specific double-labelled probe *Giardia*-105T (Biolegio, Malden, the Netherlands). They were used at a final concentration of 400 nM each for the forward and reverse primers and 120 nM for the probe in 25 µl reaction containing 5 µl template DNA (Table 2.11).

The assay incorporated an internal/external control of green fluorescent protein (GFP) primers and probe (Table 2.10) which consisted of forward primer gpfF, reverse primer gpfR, and double-labelled probe 2gpfROX at final concentrations of 300 nM, 300 nM, and 100 nM respectively (Murphy et al., 2007). The amount required in each reaction tube was worked out in a previous exercise using internal control validation data (S.D. Polley, personal communication, October 10, 2010). A sample is deemed positive for GFP control amplification if it has a Ct value ≤ x. Where x = mean Cts (for the negative extraction control and patient samples + 1.23 std. dev. Cts of patients' samples). Cts ≤ x are acceptable result and Cts > x require a 1 in 10 dilution of the sample for the assay to be repeated to counteract any inhibitor effect. When thus diluted, a positive Ct result is considered to be less than or the same as the mean of all the Cts plus 5 since the Cts will be expected to go up because of lesser amount of DNA present as a result of the dilution factor. These parameters were set experimentally to exclude 5 % of the data points (95 % CI). The cut-off Ct value for *Giardia intestinalis* was 40. This means that, any patient sample with a Ct value greater 40 will be considered as a negative result.

Amplification was performed in a Rotor-Gene Q6000 (Corbett Life Sciences) using TaqMan(R) environmental master mix 2.0 (Applied Biosystems). The cycling conditions were: Hold: 95 ^{o}C for 10 min, (Step 1: 95 ^{o}C for 15 s, and step 2: 60 ^{o}C for 60 s), steps 1 and 2 repeated for 45 cycles. A sample was recorded as positive for *Giardia intestinalis* DNA when the detected fluorescence on the red channel was greater than 0.5 (as determined by

Rotor-Gene Q software series 2.02 (Build 4), with a Ct ≤ 40 (cut off value)). Also the GFP had Cts not above the average of the GFP Cts for the run (excluding the positive control) plus 1.23. If the figure was more than this, the sample was repeated with a 1 in 10 dilution (according to internal validation data). The test result for the 1 in 10 dilution was accepted if the GFP Ct was not above the average of the GFP Cts for the run (excluding the positive control) plus 5.0.

Table 2.11: Cocktail for Verweij real-time PCR.
Each item was multiplied by the total number of tests including controls to get the required volume of cocktail for the PCR run.

gF1R1 Primers and probe	1.9 µl
GFP Primers and Probe	1.9 µl
H2O	3.7 µl
Environmental mastermix	12.5 µl
Cocktail µl per tube	20 µl
DNA µl per tube	5 µl

2.2.6 Conventional PCR simulation of Verweij real-time PCR

Conventional PCR (CPCR) simulation of the Verweij real-time PCRfor the 62 bp amplicon of *Giardia intestinalis* was performed on apparent false positive samples where the real-time positive result could not be confirmed by the composite reference standard or any of the other test results. The same reagents used for the real-time PCR were used for the conventional PCR including, *Giardia*-80F and *Giardia*-127R primer pairs, but without the probe. A G-Storm thermocycler (Kapa Biosystems Model GS00001) was used with the following protocol: Heated lid 110 ^{o}C; Hot start 95 min (for 1 cycle) and 15min (for 1 cycle); (95 ^{o}C for 45 s and 60 ^{o}C for 1min 30 s) repeated for a total of 45 cycles; 72 ^{o}C for 7 min (for 1 cycle) followed by a holding temperature of 10 ^{o}C. The amplification protocol was repeated, with the same set of primers, as a

two step reaction to maximize DNA yield. Sixty two bp amplicons were visualized using gel electrophoresis (Figure 3.4 and Figure 3.5).

2.2.7 Conventional nested PCR

A nested PCR was run with two different sets of primers to amplify the SSU-rRNA 130 bp fragment of *Giardia intestinalis.* These primers have been investigated and found to offer the best combination for a conventional nested PCR (Nantavisai et al., 2007). The conventional nested PCR was also run to investigate further the apparent false positive results. The nested PCR used two different sets of primers to amplify the SSU-rRNA 130 bp fragment of *Giardia intestinalis* for visualization using gel electrophoresis (Nantavisai et al., 2007). The primers used were RH11/RH4 and GiarF/GiarR (Hopkins et al., 1997; Nantavisai et al., 2007) (Table 2. 9).

In the nest 1 reactions, the RH11/RH4 amplified a 292-bp region of the 5' end of the (SSU) rRNA gene. The amplification was performed in 25 µl volumes with the final mix containing 5-50 ng DNA as per published method (Hopkins et al., 1997) using Biomix red (Bioline product), a complete ready-to-use 2x reaction mix containing an ultra-stable Taq DNA polymerase. The amplification process consisted of 95 ^{o}C for 2 min (1 cycle); (94 ^{o}C 20 s, 59 ^{o}C 20 s, and 72 ^{o}C 30 s) repeated for 40 cycles; 72 ^{o}C for 7 min (1 cycle) and the cocktail (reaction mix) was prepared as follows:

Nest 1 cocktail	µl/test
Biomix Red	10
RNAse/DNAse free water	7
Rh4 + Rh11 (10 µM)	1
Final volume	18
DNA	2
Total reaction vol.	20

The nest 2 primers which identified 130bp fragments of *Giardia intestinalis* were GiarF and GiarR (Read et al., 2002) (see below). A Thermo electron Px2 thermal cycler was used with the following amplification protocol: 95 ^{o}C for 2 min (1 cycle); (94 ^{o}C 20 s, 59 ^{o}C 20 s, and 72 ^{o}C 30 s) repeated for a total of 45 cycles; 72 ^{o}C for 7 min (1 cycle) as per published protocol using Biomix red (Hopkins et al., 1997; Read et al., 2002). The cocktail (reaction mix) was prepared as follows:

Nest 2 cocktail	µl/test
Biomix Red	10
RNAse/DNAse free water	7
GiarF + GiarR (10 µM)	1
Final volume	18
DNA	2
Total reaction vol	20

2.3 Results

The range of the serial dilutions of *Giardia intestinalis* cysts used did not allow the detection of a cut off point for the Verweij real-time PCR. The limit of detection (LOD) was therefore estimated to be ≤ 4.5 cysts/ml (< 5 cysts/ml). The LOD for the Primerdesign Ltd. PCR was ≤ 113.6 cysts/ml (≤ 114 cysts/ml) (Table 2.12). With the exception of an isolated positive result (Ct 38.9) in one of the triplicate run in tube 6, the Primerdesign Ltd. assay did not detect any positive in the rest of the tubes from 6 to 7 and therefore the LOD was estimated to be ≤ 114 cysts/ ml. The LOD for the CRS was assessed to be ≤ 2840 cysts/ml and analytical specificity was 100 % for each of the diagnostic test evaluated because no cross reaction was detected with any of the other microbial flora tested in the pooled *Giardia*-negative stool.

At an estimated figure of < 5 cysts/ml, the Verweij real-time PCR had the highest analytical sensitivity compared to the other tests.

Table 2.12: Determination of limit of detection (LOD) using cysts.
***Giardia intestinalis* cysts were used and the Verweij real-time PCR did not have a cut-off (LOD < 4.5 cysts/ml) and all the tests were analytically specific, i.e. no positive results was obtained with the pooled *Giardia*-negative stool.**

Test	Tube nos.							Pooled *Giardia*-negative stool
	1	2	3	4	5	6	7	
RMT	+	-	-	nt	nt	nt	nt	-
OCP-M	+	+	-	-	-	-	-	-
EIA	+	+	+	-	-	-	-	-
Primerdesign	+	+	+	+	+	-	-	-
Verweij	+	+	+	+	+	+	+	-
Cysts/ml	71000	14200	2840	568	113.6	22.7	4.5	

nt = not tested; + = Positive; - = Negative.

Detection limits for *Giardia* trophozoites were also assessed for all the diagnostics tests including the EIA and RMT which formed the CRS (Table 2.13).

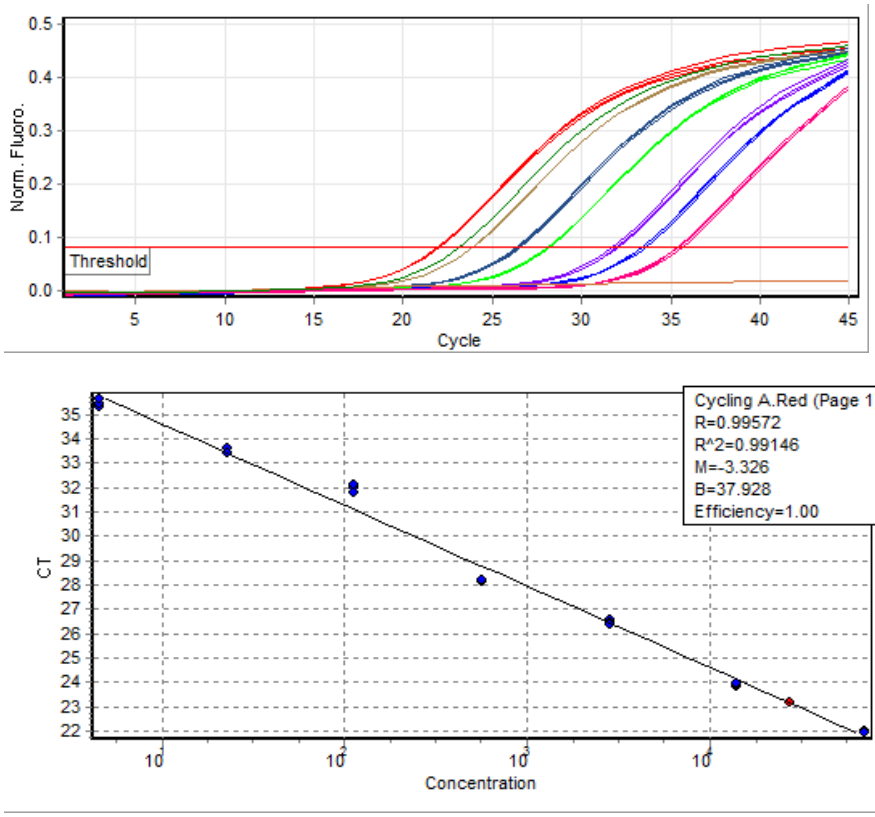
Table 2.13: Determination of detection limits using trophozoites.
Antigen detection test performed better than DNA detection tests. No cross reactions were detected in any of the tests listed.

Test	Tube nos.							Pooled *Giardia*-negative stool
	1	2	3	4	5	6	7	
OCP-M	+	-	-	-	-	-	-	-
Primerdesign	+	-	-	-	-	-	-	-
Verweij	+	+	-	-	-	-	-	-
RMT	+	+	-	nt	nt	nt	nt	-
EIA	+	+	+	+	-	-	-	-
Trophozoites/ ml	92000	9200	920	92	9.2	0.92	0.092	

nt = not tested; + = Positive; - = Nagative.

Antigen detection using EIA was the most analytically sensitive test for trophozoites with the estimated LOD of $\leq$ 92 trophozoites/ml. In practice, however, the PCR assays do not differentiate between DNA from trophozoites and DNA from cysts.

A



B.

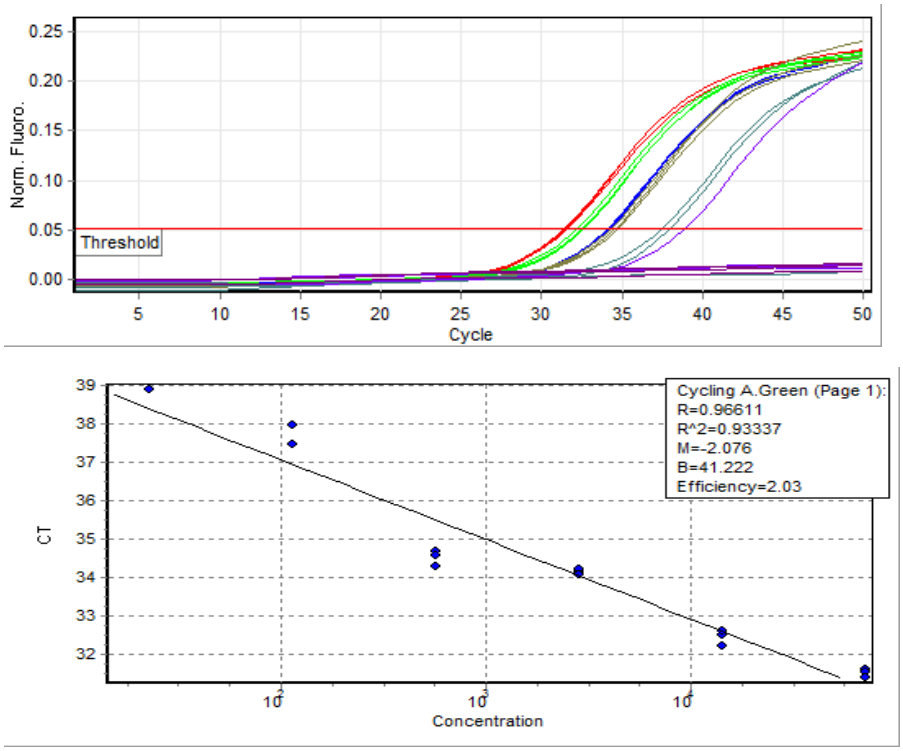


Figure 2.7: Amplification graph and standard curve constructed using the 1 in 5 serial dilutions of the *Giardia* positive stool.

The stool sample employed in the analytical sensitivity testing (i.e. Limit of detection (LOD)) in Table 2.1 was also used for this standard curve. The data is presented here in a comparative sense only for the LOD between the Verweij real-time PCR (A) which was estimated to be < 5 cysts/ml of stool and the Primerdesign Ltd. real-time PCR (B) which was also estimated to be ≤114 cysts/ml. Further verification was warranted because of the apparent differences in amplification efficiencies and tests reproducibility. Both "A" and "B" tests were performed in triplicate.

Using clinical samples, the Primerdesign Ltd. real-time PCR standard curve produced an efficiency of 203 % (the slope was -2.076) (Figure 2.7 B) whilst the Verweij real-time PCR standard curve gave an efficiency of 100 % (the slope is -3.326) (Figure 2.7 A).

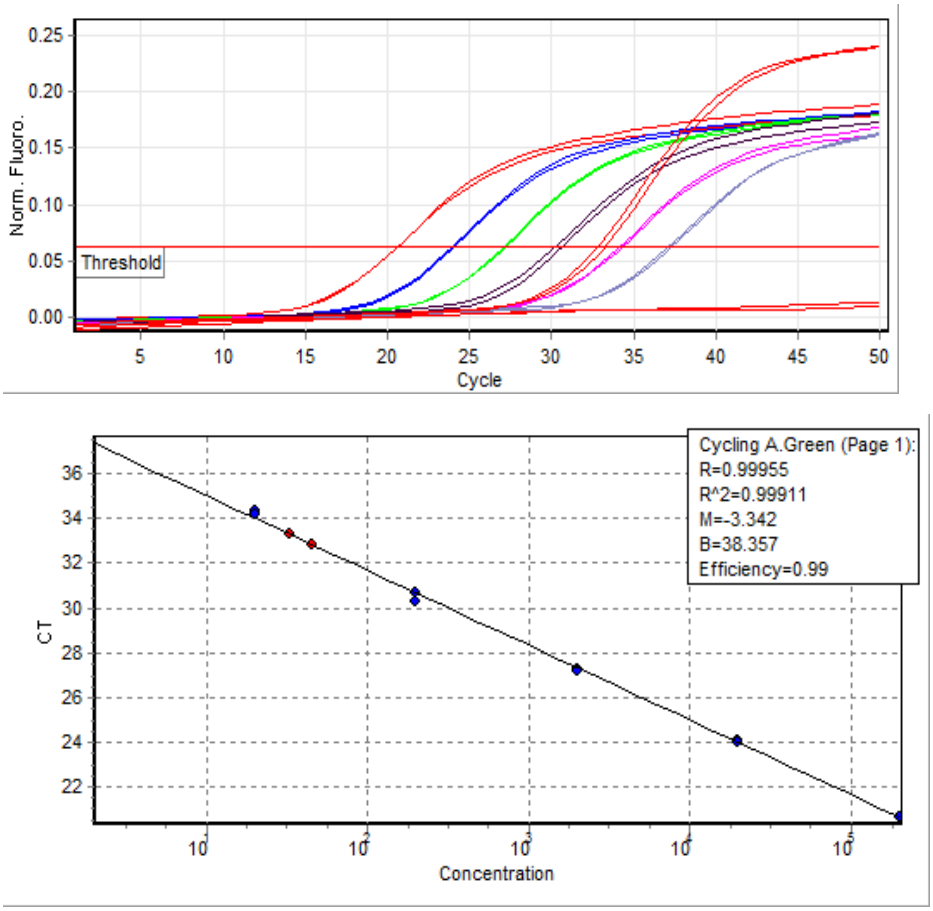


Figure 2.8: Primerdesign Ltd. PCR amplification graph and standard curve. These were constructed using 1 in 10 serial dilutions of the *Giardia* DNA positive control sample supplied with the Primerdesign Ltd. real-time PCR kit. The tests were performed in duplicate and the data showed a better performance over the clinical samples presented in Figure 2.7 B producing a 99 % efficiency and R^2 value 1.00

Generally an efficiency between 90 % and 110 % is considered acceptable (Real-time PCR: Understanding Ct, 2011). The serially diluted positive control from the Primerdesign Ltd. kit (purified *Giardia intestinalis* DNA solution), however, produced an efficiency of 99 % and R^2 value of 1.00 using the same environmental mastermix as was used for the Verweij real-time PCR (Figure 2.8). Clinical samples (stools) are a more challenging material because of the host of microbial flora, faecal matter and PCR inhibitors that they contain. The Verweij real-time PCR with efficiency of 100 % on clinical sample, $R^2 = 0.99$ and LOD of < 5 cysts/ml has shown robustness in performance compared with the Primerdesign Ltd. PCR.

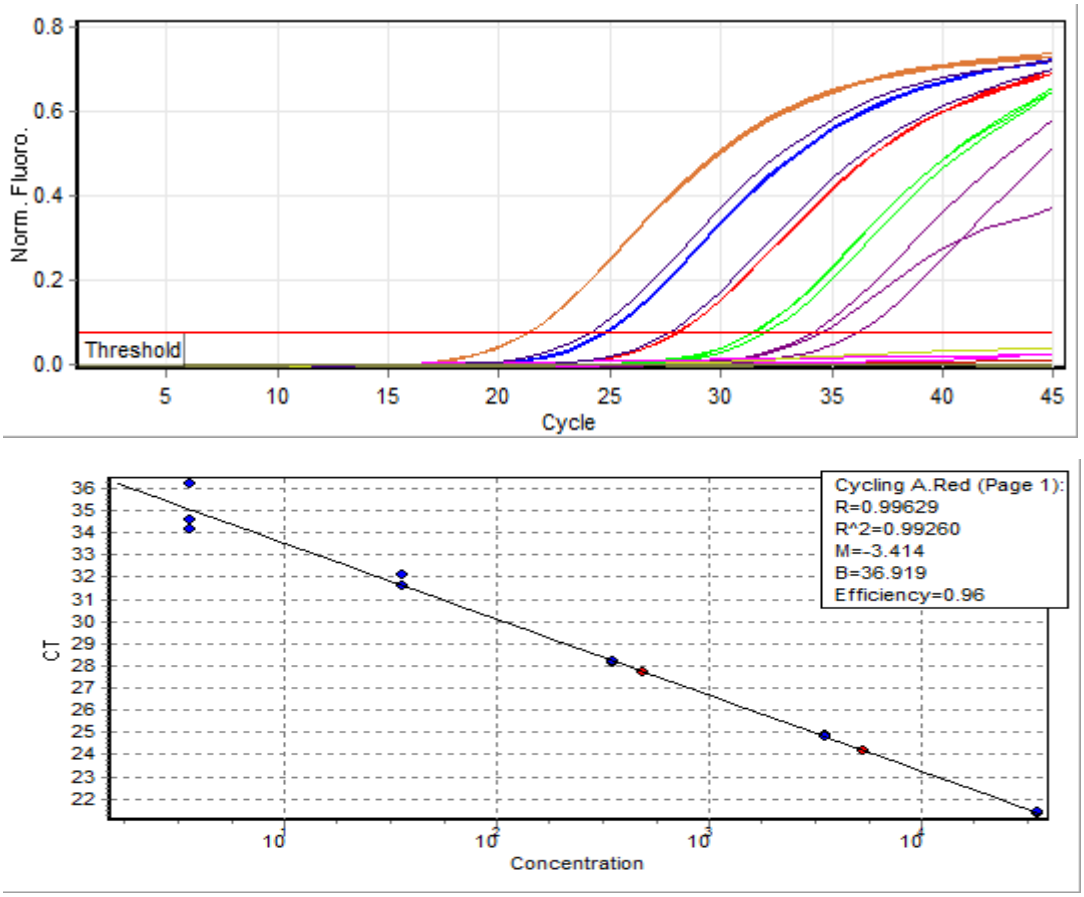


Figure 2.9: Verweij real-time PCR run using 1 in 10 dilution of a DNA solution with known concentration.
Efficiency was 96 % with an R^2 value = 0.99. *Giardia* DNA was detected in all five tubes.

The kit positive control appears to have been optimised by the manufacturer hence the better result compared with the 203.3 % obtained from clinical samples. This assumption was further investigated with a repeat run of the two real-time PCR assays.

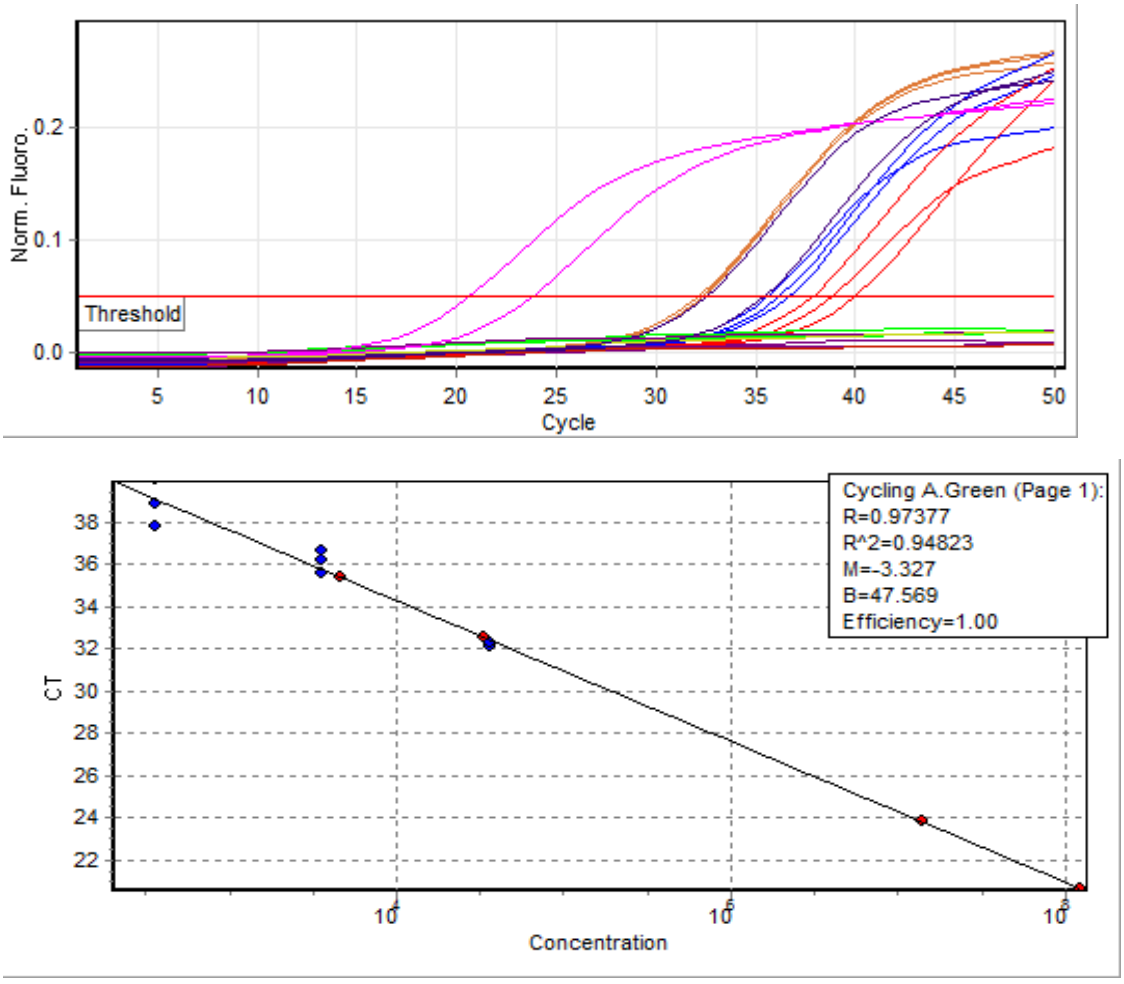


Figure 2.10: Primerdesign Ltd. real-time PCR run: One in 10 dilution of the DNA solution with known concentration was used.
Efficiency was 100 % with an R^2 value = 0.95. Data points were from three out of the five serial dilutions as *Giardia* DNA could not be detected in tubes 4 and 5 representing 35.5 cysts/ml and 3.55 cysts/ml respectively. R^2 indicates how good threshold cycle values are at predicting concentration values. R^2 value > 0.99 provides good confidence in correlating two values. If R^2 = 0 the value of a sample cannot be predicted from the other. R^2 recorded for this PCR run was 0.95.

One in 10 serially diluted *Giardia* DNA solutions obtained from Table 2.1, Tube no. 1 was employed. Figure 2.9 and Figure 2.10 show the

amplification graphs and the standard curves obtained for both PCR runs and the Table 2.14 and Table 2.15 provide a summary of the findings from the assays' performances with the percentage variance (% var) between the given concentrations of cysts or copies/ ml. and the calculated equivalent also shown.

Table 2.14: A summary of results for the analytical verification (1). The Verweij and the Primerdesign Ltd. real-time PCR assays were tested with DNA extracted from 1 in 5 dilutions of a *Giardia* positive stool sample.

Assay parameters	**Dec 2011:** First testing of DNA extracted from 1 in 5 dilutions of stool. Test performed in triplicate		Purified *Giardia* DNA
	Verweij	Primerdesign Ltd.	Primerdesign Ltd.
LOD	< 5 cysts/ ml	≤ 114 cysts/ ml	≤ 2 copies/µl
Efficiency (%)	100	203	99
R^2	0.99	0.93	1.00
Slope	-3.326	-2.076	-3.342
Variance (% Var)	**Predominantly > 10 %Var** i.e. (66.7 %)14 samples > 10 % Var (33.3 %) 7 samples < 10 % Var All 21 samples detected (100 %)	**Predominantly > 10 %Var** i.e. (57.1 %)12 samples > 10 % Var (14.3 %) 3 samples < 10 % Var 6 out of 21 not detected (28.6 %)	**Predominantly < 10 %Var** i.e. (58.3 %)7 samples < 10 % Var (41.7 %)5 samples > 10 % Var All 12 samples detected (100 %)

It is clear from the data in Table 2.14 and Table 2.15 that all of the Primerdesign Ltd. Ct values, for both the extracted stool and the diluted DNA from the stool showed a variance predominantly > 10 %. A variance ≤ 10 % is generally considered acceptable ((Dhanasekaran, Doherty, Kenneth, & Group, 2010). Also R^2 in both instances was < 0.99. The Verweij real-time PCR showed variance predominantly > 10 % with the extracted stool (Table 2.14). The figures improved in Table 2.15 when the 1 in 10 serially diluted DNA from the stool were tested producing a variance predominantly < 10 % in the

preferred range of values. R^2 values were however consistently 0.99 in both cases of testing.

Table 2.15: A summary of results for the analytical verification (2).
The Verweij and the Primerdesign Ltd. real-time PCR assays were tested with 1 in 10 serially diluted DNA solutions from Table 2.1 about 20 months after it was first tested.

Assay parameters	**July 2013:** Second testing (20 months after the first) of 1 in 10 dilutions of DNA solution. Tested in triplicate	
	Verweij	Primerdesign Ltd.
LOD (cyst/ml)	< 3.55	≤ 355
Efficiency (%)	96	100
R^2	0.99	0.95
Slope	-3.414	-3.342
% Var	**Predominantly < 10 % Var** i.e. (73.3 %) 11 samples < 10 %Var (26.7 %)4 samples > 10 % Var All 15 samples detected (100%)	**Predominantly > 10 % Var** i.e. (13.3 %) 2 samples < 10 % Var (46.7 %) 7 samples > 10 % Var 6 out of 15 not detected (40 %)

The purified *Giardia* DNA in the Primerdesign Ltd. real-time PCR kit gave excellent results with the kit's primers targeting the gdh gene of *Giardia intestinalis.* Efficiency was 99 %, $R^2 = 1.00$, and LOD ≤ 2 copies/ml with a positive detection in all tubes of the dilution series in sharp constrast with the performance observed when a clinical (stool) sample was used (Table 2.14). Primerdesign 2x precision mastermix was also used with the Primerdesign Ltd. kit to retest 36 *Giardia intestinalis* positive samples previously tested positive with environmental mastermix. Out of the 36 samples, only one retested positive with a Ct of 22.85 (sample 71, Burkitts lymphoma case). *Giardia intestinalis* trophozoites only were seen on microscopy. The environmental mastermix performed better than the Primerdesign 2x precision mastermix and the latter was not used again anywhere else in this project.

2.4 Discussion

The purpose for this chapter was to ascertain the analytical sensitivity (limit of detection) and specificity of the diagnostic tests deployed in this study in order to assess their suitability for diagnostic accuracy studies. The two main constituent tests of the CRS detected antigens of *Giardia intestinalis*, and the PCR assays detected 62 bp and 130 bp fragments of *Giardia intestinalis* DNA. It was only the OCP-M that detected whole parasites instead of components of the parasite.

In general, analytical sensitivity was better for most of the diagnostic tests when cysts instead of trophozoites were used (Table 2.12 and Table 2.13). The OCP-M for example used about the equivalent 10 µl of liquid stool to prepare thin faecal smears which were stained for the visualization of *Giardia intestinalis* trophozoites and a drop of about 50 µl stool was used for wet preparation examination for the trophozoites. These volumes are relatively small and only stools with very high counts of *Giardia* trophozoites will produce positive results. The OCP-M does not concentrate trophozoites because the organic solvent (ethyl ether) used tends to destroy them and therefore only cysts were seen from the 1 ml of stool that was concentrated hence the better analytical sensitivity performance of cysts over trophozoites. The fatty plugs in the faecal concentration method are known to trap not only helminth ova but also parasite cysts to different degrees according to the organic solvent used (ethyl acetate or ethyl ether) and whether a preservative was added to the stool or not prior to performing the concentration technique. The addition of additives affect the specific gravity of fluid in the mixing chamber of the Parasep and subsequently the OCP-M sensitivity therefore centrifugal speed and timing need to be set accordingly (Saez, Manser, Andrews, & Chiodini, 2011; Zeeshan et al., 2011).

The RMT detected *Giardia* cyst membrane bound antigens and the test was not designed to be used on trophozoites. However, compared with the

OCP-M, there was only one dilution tube difference between both tests in respect of the detection of both cysts and trophozoites (Table 2.12 and Table 2.13). Their analytical sensitivity test performances were therefore similar howbeit low. The RMT had to be repeated on occasion because of poor diffusion of the fluid gradient on the membrane strip. If this was an inherent weakness in the design of the strip, that could explain its low analytical sensitivity. Availability of membrane bound antigens relies on the presence of cysts and in a situation where cyst numbers are low, detection of the parasite will be difficult because of the small sample volume tested (10 - 20 µl stool).

Contrary to the RMT, the EIA detected *Giardia* soluble cyst wall antigens and performed better on trophozoites than cysts (Table 2.12 and Table 2.13). Encysting trophozoites secrete these antigens and it could be envisaged that in the diluents, antigens were secreted in an attempt to encyst hence their detection in the trophozoites dilution tube 4 (≤ 92 trophozoites/ml) at a higher titre compared with the ≤ 2840 cyst/ml in the cysts dilution tube 3. The EIA detected soluble antigens and performed better than the RMT. The test can therefore be performed even in the absence of cysts on microscopy because soluble antigens could still be floating around. Similarly, the EIA performed better on trophozoites than the two real-time PCRs suggesting that soluble antigen detection could be the better choice of test to use instead of DNA detection in situations where trophozoites are more likely to be encountered (e.g. as in duodenal aspirates). The assessed EIA detection limit for trophozoites (≤ 92/ml) suggested the possibility that any stool sample which had less than 92 *Giardia* parasites may not have been detected by the CRS because the EIA was relatively more sensitive than the RMT in the formation of the CRS. This meant that any index test more sensitive than the CRS will have more false positive results which would impact on its specificity.

The Verweij real-time PCR was more analytically sensitive than the Primerdesign Ltd. real-time PCR with tests performed on both the cysts and

trophozoites of *Giardia intestinalis*. This is a reflection on the gene target used in both tests. The (SSU) rRNA targeted by the Verweij real-time PCR has been found to be more conserved with less variability and therefore it is used more for genotyping (Feng & Xiao, 2011). The gdh target for the Primerdesign real-time PCR has been reported to be relatively less conserved and therefore used for both genotyping and sub-typing (Feng & Xiao, 2011).

The DNA detection in cysts was not only superior to the detection of antigen in cysts; it was also superior to the detection of it in trophozoites as indicated by the LOD's (Table 2.12 and Table 2.13). There appeared to be a lot more *Giardia* DNA present that would not be titred out using the Verweij real-time PCR and in a way showed how sensitive the Verweij real-time PCR was. The reason for this could be the fact that *Giardia intestinalis* show genome polyploidy in their chromosome arrangement. Polyploid cells and organisms are those containing more than two paired (homologous) sets of chromosome (Bernander, Palm, & Svärd, 2001). *Giardia* trophozoites go through two successive rounds of chromosome replication without cell division event. A fully differentiated cyst contains four nuclei, each with a ploidy of 4N, resulting in a cyst ploidy 16N (Bernander et al., 2001). The formation of multiple cells from a single cyst is likely to be one of the main reasons for the high titre in the serial dilutions of the DNA compared with the antigens because of the quadrupling of genome ploidy. Between the two real-time PCRs, the Verweij real-time PCR had a lower detection limit than the Primerdesign Ltd. PCR. In particular, there was no cut off limit for the detection of cysts using the dilutions employed in the LOD test for both real-time PCRs. The estimated Verweij PCR LOD was < 5 cysts/ml of stool and that for the Primerdesign Ltd. was ≤ 114 cysts/ml (Table 2.12).

With almost every step the same for both assays from sample processing to the reading of the results, the differences in the analytical measures could only be attributed to the gene targets for the respective primers. The gene target for

the Primerdeisgn Ltd. PCR was the gdh gene and that for the Verweij real-time PCR was the (SSU) rRNA. It is known that the sensitivity of PCR amplification of the gdh gene is limited, in particular, when low numbers of cysts are present in faecal samples (Bertrand et al., 2005; Nantavisai et al., 2007). There was no positive *Giardia intestinalis* DNA detected when cysts count fell below the estimated 113.6 per ml (Table 2.12). This was in contrast with the real-time PCR SSU-rRNA primers *Giardia*-80F and *Giardia*-127R for there was no end point reached in the range of serial dilutions used in this study. The (SSU) rRNA gene is therefore a more reliable target to use as a first line test for the diagnosis of giardiasis. Asher et al. (2012), will however disagree with this conclusion because in a study to evaluate a PCR protocol for the detection of *Giardia intestinalis*, they reported a success rate of 90 % for (18S) rRNA and 94 % for the gdh loci (Asher, Waldron, & Power, 2012). They amplified a 130 bp product from the (18S) rRNA gene using the primers RH11 and RH4 as previously described (Hopkins et al., 1997) and the primers GiarF and GiarR for the secondary PCR as previously described (Read et al., 2002). They however reported extracting the DNA directly from only 50 mg of stool with no pre-washing of the samples. This could account for the result that they got whereby gdh appeared to be the better loci to use for *Giardia* detection. In this study, 500 mg of stool was pre-washed to remove soluble PCR inhibitors before DNA was extracted. The larger amount of stool used together with the pre-washing could account partly for the difference in performance. The LOD results therefore have shown an increased sensitivity level when the (SSU) rRNA is the target instead of the gdh and also underlines the importance of stool washing to reduce the effect of PCR inhibitors before extraction.

In this study, the effect of PCR inhibitors was also seen in the improvement of efficiency for the Primerdesign Ltd. real-time PCR from E = 203 %, R^2 = 0.93 when DNA extracted directly from stool was tested to E = 100 %, R^2 = 0.95 when the extracted DNA was serially diluted 1 in 10 and retested 20 months

later (Table 2.14 and Table 2.15). The inhibitor concentrations/ effect were lowered by the serial dilutions and this reflected in the reduction of percentage variance (% var) figures. Test results with % variance > 10 % dropped from 12 samples when first tested with DNA extracted directly from stool down to 7 samples when tested with the 1 in 10 dilutions from the serially diluted DNA solution (Table 2.14 and Table 2.15). Overall degradation of DNA was not apparent from the set of results obtained after the 20 months storage at -20 ^{o}C (Table 2.14 and Table 2.15). The improvement in efficiency, notwithstanding, the Primerdesign Ltd. real-time PCR was not robust or sensitive enough to detect *Giardia* DNA in low concentrations even in the diluted DNA solution from the stool sample with reduced inhibitory substances. LOD ≤ 355cysts/ml was estimated (Table 2.15). For reasons already alluded to, the gdh gene targeted by the Primerdesign Ltd. real-time PCR is less sensitive and more discriminatory than the (SSU) rRNA and therefore it is capable of sub-typing of (Feng & Xiao, 2011). Genotyping at the (18S) rDNA locus is based on a relatively small amount of sequence data (e.g. 130 bp) compared to the 450 bp amplified at the *gdh.* The latter provides more data making it perhaps a more reliable assay on which to assign genotype/ sub-types (Read et al., 2004).

Usually primers giving 60 bp to 150 bp amplicon sizes are considered ideal for a reliable PCR efficiency. Amplicons more than 150 bp may give < 100 % efficiency especially if they have not been designed to span exon-exon junctions giving rise to genomic DNA amplification and primer dimer formation (Dhanasekaran et al., 2010). With the exception of two out of the three Primerdesign Ltd PCR Tube 1 replicates showing % var < 10, increased variance, 13.7 to 138.8 %, were seen in all the standard solutions irrespective of whether the concentrations were low or high. The very low concentrations in tubes 4 and 5 (Table 2.1) were not detected at all.

The Verweij real-time PCR efficiency dropped slightly from 100 % down to 96 % but stayed in the acceptable range of 90-100 %. The assay was robust

enough to detect *Giardia* DNA in all dilutions at both instances when the assay was performed.

R^2 indicates how good one value is at predicting another. Usually 0.99 and above provides good confidence in correlating two values. The Verweij real-time PCR gave R^2 value 0.99 when it was first tested and again 0.99 when tested after 20 months. The figures for the Primerdesign Ltd. PCR (0.93 and 0.95), however, never got up to 0.99 on both occasions of testing (Table 2.14 and Table 2.15) indicating yet again the superiority of the Verweij real-time PCR over the Primerdesign Ltd. one. Copy number variations are known to occur as result of degradation of target sequences, which in turn can directly affect PCR efficiency but not the correlation coefficient R^2 (Dhanasekaran et al., 2010). It has therefore been suggested that PCR efficiency, copy number variance and correlation coefficient R^2 are equally important for quantification (Dhanasekaran et al., 2010). The two main reasons why these three parameters are equally important is 1) pipetting variations and 2) the large molecule of DNA. In this study, 20 months of DNA storage did not appear to have adversely affected PCR efficiency. DNA degradation, if any, was minimal judging from the efficiency figures (Table 2.14 and Table 2.15). Pipetting variations could also be the reason behind the > 10 % variations and not just PCR inhibitors alone. DNA molecules are large and they are likely to make interactions with other molecules as well as intramolecular (electrostatic) interactions. The molecules do not behave like smaller molecules in solution and the interactions are hard to predict (Mygind et al., 2002). It has been suggested that for gene expression studies, primers targeting 60 bp to 150 bp amplicon sizes are considered ideal because > 150 bp amplicon sizes may give < 100 % PCR efficiency(Dhanasekaran et al., 2010). This could explain why the variances in the Verweij real-time PCR and the Primerdesign Ltd. real-time PCR showed a Predominantly > 10 % but the Verweij real-time PCR alone showed a variance predominantly < 10 % after the DNA was serially diluted but Primerdesign Ltd.

real-time PCR remained predominantly > 10 % when the same set of dilutions were tested. The 62 bp amplicon sizes targeted by the SSU-rRNA primers of the Verweij real-time PCR appeared smaller than the gdh target (Read et al., 2004) The latter DNA sequence has not been disclosed by Primerdesign Ltd. and all attempts to amplify the DNA by using it as one of the positive controls for the Verweij real-time PCR failed. Even though the performance figures for the Primerdesign Ltd. PCR using the provided *Giardia* positive control is excellent (E = 99 %, R^2 = 1.00, LOD ≤ 2 copies/ µl, and % var = Predominantly 10 %), it has been optimised for the kit and does not represent the harsh environment of clinical faecal samples. The information derived from the test run has been used only in a comparative sense to highlight the effect of PCR inhibitors and accuracy in pipetting.

It is also known that the detection very low copy numbers do not follow normal distribution of template but rather a Poisson type which stipulates that in a large number of replicates with an average of one copy of starting template, approximately 37 % will have no copies, 37 % will have one copy, and 18 % will have two copies (Real-time PCR: Understanding C_t, 2011). Thus in this study the 1 in 10 dilutions for standard curves were tested in triplicate to overcome the Poisson distribution limitation (Figure 2.9 and Figure 2.10).

The OC&P method is not designed for trophozoites because the organic solvents used in the procedure tends to destroy them. However, direct smears from the unconcentrated stool can be prepared and stained with rapid Field's stain for *Giardia* trophozoites and also wet preparations can be prepared to look for live *Giardia* trophozoites. Even with the improved OCP-M, the analytical sensitivities for both trophozoites and cysts obtained in this study were relatively very low with detection limits of ≤ 92,000 trophozoites/ml of stool and ≤ 14,200 cysts/ml stool respectively (Table 2.12 and Table 2.13). The OCP-M method uses ethyl ether as an extractor of fat and debris from stools. The advantage of this method is that it will recover most ova, cysts and larvae and

retain their morphology thus facilitating their identification; it is not for *Giardia* species only. This method can be used on fresh as well as preserved samples, e.g. samples which have been preserved in formalin, sodium acetic acid formalin (SAF), and polyvinyl alcohol (PVA). It however has the disadvantage of destroying trophozoite stages and distorting cellular exudates. Also, mucoid stools do not concentrate well and therefore it will be necessary in these cases to examine the stool by direct microscopy instead of the concentration method. Cysts of *Giardia intestinalis* can be trapped in the ethyl-fatty plug and be lost. It is not surprising therefore that the assessed LOD for the OCP-M was very high, ≤ 14,200 cysts/ml. The RMT test was not performed on samples containing trophozoites from tube 4 to tube 7 and cysts from tube 4 to 7 because the test result had already gone negative by tube 3 in both cases. About three of the RMT strips were repeated because of unsatisfactory diffusion of fluid on the strips. This was caused by the mucoid consistency of the stool impeding the lateral flow of the stool and buffer suspension. Vortexing the faecal suspension for longer resolved the situation.

So far, of the five tests investigated, the EIA gave the best detection level of ≤ 92 cysts/ml. The EIA detects soluble antigens released into the stool environment by *Giardia intestinalis* particularly when encystation is taking place. These results are exploratory as they were meant to find a baseline for comparing the tests for when the diagnostic sensitivities and specificities are tested. The detection limits with cysts showed the superiority of PCR over the antigen detection tests of the RMT and EIA, and also over the OCP-M.

Analytical specificity for these tests were also investigated and no cross reactivity was detected in any of the tests when used on a pooled *Giardia* negative stool sample containing a host of faecal microbial flora. If a test has a high Analytical sensitivity it does not automatically mean that it will have a high diagnostic sensitivity and/or specificity(Saah & Hoover, 1997). The next section will show how the analytical sensitivities of the Index tests translated

into diagnostic sensitivities and specificities. The CRS LOD for trophozoites was good but with an estimated LOD of ≤ 2840 cysts/ml, it will be difficult to detect low numbers of *Giardia* cysts and considering that cysts forms of *Giardia* are found in stools more often than trophozoites, false positive results will have to be investigated further in the verification exercise in the next chapter.

Chapter 3: Verification of diagnostic sensitivities and specificities of tests for *Giardia intestinalis*

3.1 Introduction

The choice of the gold standard in any diagnostic test verification is crucial for the determination of true positive and true negative cases. Microscopy is considered to be the gold standard for the diagnosis of giardiasis but it is an imperfect gold standard and the sensitivity of parasite detection rarely gets to 90 % due to the intermittent pattern of excretion of the parasite which can lead to their sub-optimal levels that cannot be detected using the direct smear or concentration method (Duque-Beltrán et al., 2002). Even when multiple samples taken on different days have been examined, a much improved sensitivity of parasite identification has been reported to be only 85 % (Duque-Beltrán et al., 2002; Gaafar, 2011). To avoid the risk of bias and to ensure the quality of this diagnostic research, the current version of the quality assessment of studies (QUADAS) tool, i.e. QUADAS 2, was consulted in the design of this diagnostic accuracy study. The tool is recommended in systematic reviews for the assessment of the risk of bias and sources of variation in studies (Whiting et al., 2011). From the tool the following information were used to design this diagnostic accuracy study:

1. A composite reference standard was used in the absence of a gold standard avoiding incorporation bias with the index tests by selecting different targets for the tests.

2. Bias in the selection of samples was avoided by using a consecutive non-probability sampling technique.
3. Second qualified (biomedical scientists) readers who had no knowledge of the results were used to avoid workup bias, and
4. All the study samples were individually tested with each of the index tests thereby avoiding verification bias

A composite reference standard (CRS) of RMT and EIA was used in the comparative study of the three index tests, viz. OCP-M, Primerdesign Ltd. real-time and Verweij real-time PCR assays. The procedure has been recommended by the Health Technology Assessment (HTA) for studies when there is no gold standard (Rutjes, Reitsma, Coomarasamy, Khan, & Bossuyt, 2007). These reference tests detect *Giardia* antigens and avoid any incorporation bias with the index tests. The index tests have the intact parasite (detected by the OCP-M) and genomic DNA (detected by the real-time PCR assays) as their targets for the identification of the parasite. Some researchers have used other experimental designs that involved combinations of the index test as extended reference test in the absence of a gold standard(Elsafi et al., 2013; Schuurman et al., 2007).

Whilst QUADAS 2 deals with quality issues in methodology, STARD, which stands for the Standard of Reporting of Studies, similarly deals with quality in the reporting of studies by emphasizing on its accuracy and completeness at the end of the study. To ensure accurate and full reporting of this study, a flow chart has been provided to show the outline of the investigatory path beginning with sample selection through to the results of the index tests (Figure 3.1) with explanations provided in the text. STARD format of introduction, method, result, and discussion has been adopted as the style for the reporting of this study. The STARD statement has recommended this approach to introduce clarity, accuracy and completeness of the information given in the reporting of

studies (Bossuyt et al., 2003; Standards for the Reporting of Diagnostic accuracy studies (STARD) Statement, 2008).

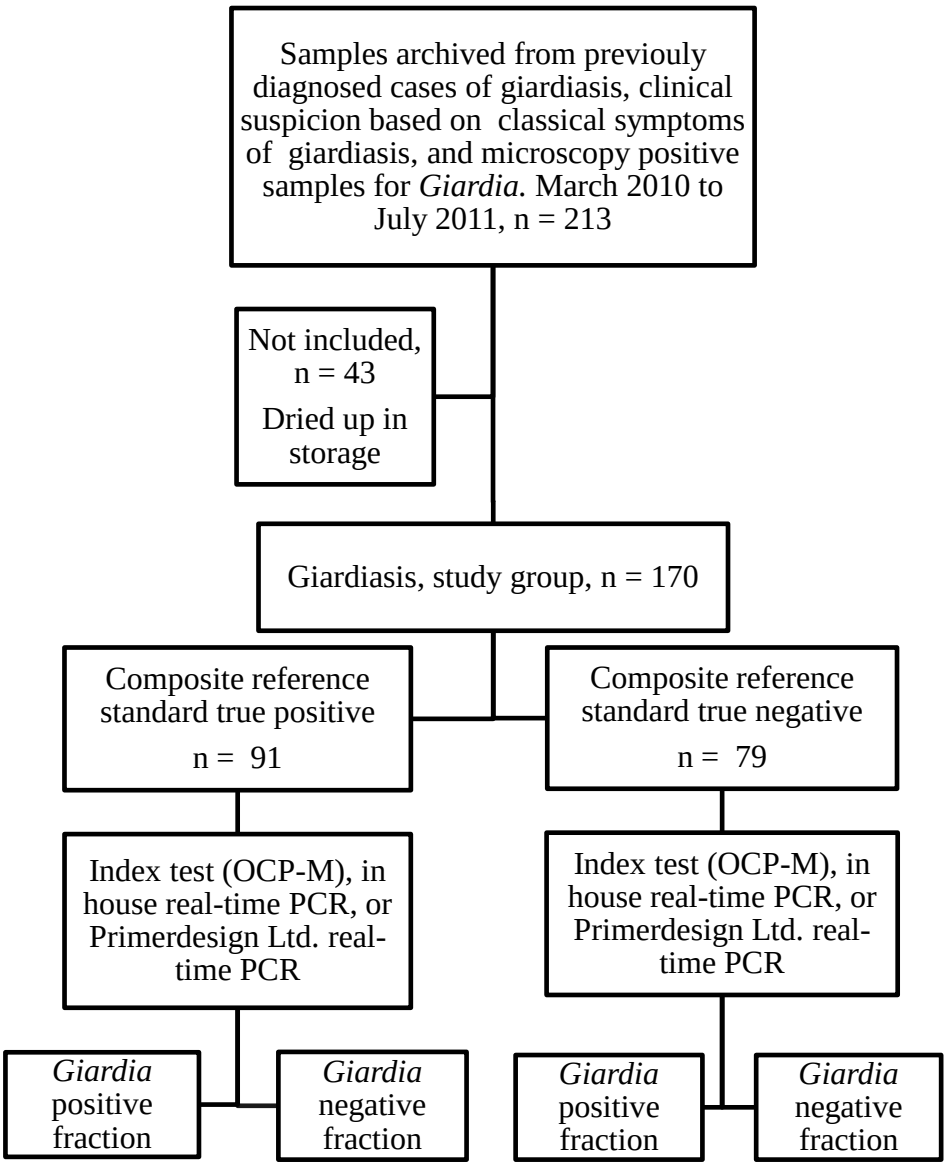


Figure 3.1: Flow chart of lines of investigation for *Giardia*.
The study group was made up of 170 samples and each sample was tested with the composite reference standard to delineate true positive and true negative cases.

Whereas STARD checks the information that ideally should be reported, QUADAS evaluates the quality of the reported information (Oliveira, Gomes, & Toscano, 2011).

3.2 Methods

The (SSU) rRNA and the gdh genes of *Giardia intestinalis* have been used in the molecular characterization of *Giardia intestinalis* (Gómez-Couso et al., 2012). Using a composite reference standard of EIA and RMT, the sensitivity and specificity of OCP-M, Primerdesign Ltd. real-time PCR and Verweij real-time PCR were compared (see Section 2.2 for how the individual tests were performed). A conventional PCR simulation of the Verweij real-time PCRand a nested PCR protocol amplifying fragments of 62 bp and 130 bp respectively of the (SSU) rRNA gene of *Giardia intestinalis*, were also performed as backup tests for the CRS to resolve any discrepant results.

3.2.1 Sample selection

All samples received for standard diagnosis by stool OCP-M in the Department of Clinical Parasitology at HTD, London were considered for inclusion in this study. A non-probability sampling technique with consecutive samples was adopted for this study. Over a 15-month period, from 30th March 2010 to 22nd July 2011, biomedical scientists processing stools for OCP-M investigations were asked to archive all samples which were positive for *Giardia intestinalis* by microscopy. In addition they were also asked to archive samples which were negative for *Giardia intestinalis* but fulfilled any of the criteria given in Table 3.1.

Table 3.1: A sample population of 213 was collected.
The OCP-M was initially used to test these samples as per routine practice but the results were revealed only after all other tests in this study were completed.

Sample Groups	Total
Clinical suspicion (specific request to test for *Giardia*)	213
Previously diagnosed *Giardia*	
Other GI disturbances where giardiasis was not singled out for investigation.	
Total	

In all, 213 samples were archived, comprising 98 *Giardia* microscopy positive samples and 115 microscopy negative samples. Each sample was split and stored at 4-6°C and at -20°C.The actual testing of these samples took about 8 months from July, 2011 to February, 2012.

Forty three samples were excluded for the following reasons: Samples with small volumes < 2 g or < 5 ml and samples dried up in storage. Also excluded were the oldest of multiple samples from the same patient. All identification labels on the 170 samples were masked and the samples were relabelled randomly from 1-170 by a biomedical scientist who had no prior information about the OCP-M results for these samples. Following the re-labelling, all the 170 samples were tested with EIA and then with RMT (composite reference standard) (Table 3.2).

Table 3.2: Delineation of true positive and negative samples.
The composite reference standard divided the 170 samples into 91 true positive and 79 true negative samples according to the four criteria listed.

Sample Group	Reference Tests		Composite Ref. Test result	No. of samples	Total
	Enzyme immunoassay (EIA)	Rapid Membrane Test (RMT)			
1	P or WP	N	TP	10	91
2	N	P or WP	TP	2	
3	P or WP	P or WP	TP	79	
4	N	N	TN	79	79
Total					170

P = Positive, WP = Weak positive, N = Negative, TP = True positive, TN = True negative

Seventy nine true negative samples were designated using the criteria given in sample group 4 in (Table 3.2) and 91 true positive *Giardia intestinalis* stools were also designated using sample groups 1 to 3 in Table 3.2 as a guide. The 170 stool samples were all individually tested with both constituent

reference tests of the CRS, and the three index tests (Tables 3.6 displays the results table).

3.2.2 Verification of power and sample size estimations

Power and sample size estimations are measures of the number of subjects enrolled in a study. They provide the number of participants required to avoid type I (false positive) and type II (false negative) errors. In a comparative study, "power" refers essentially to the number of subjects required to avoid type II error. Sample size is more encompassing and is applicable to all types of studies (Jones, Carley, & Harrison, 2003).

This research project being a comparative study and because the sample size of 170 (91 positive and 79 negative stool samples) was the available sample size fixed by the study design (see sub-section 3.2.1), it was put through sample size investigations to determine whether at 91 samples for sensitivity and 79 samples for specificity, it could support 95 % confidence limit and 0.95 power (probability) calculations. The power of the study is the probability that, given the sample sizes specified, and the expected confidence level, the study will be able to detect a predetermined difference in performance between two diagnostic tests if a difference truly exists.

The sample size interrogation was based the work of Flahault, Caldihac and Thomas (2004). They described the determination of sample size for binary diagnostic test assessment studies, and provided exact tables based on the binomial distribution and 0.95 power (see Figures 3.2 and 3.3) (Flahault, Cadilhac, & Thomas, 2005).

Before using the tables, the works of three groups of researchers were consulted (Table 3.3) for the accuracy measures that they obtained using the same SSU-rRNA primers used in this study.

Table 3.3: Sample sizes used in three independent research studies.
All three groups of workers (nos.1-3) used the SSU-rRNA primers for *Giardia intestinalis* identification with antigen detection and another G. Intestinalis-specific PCR as gold standard (used by no. 1), extended reference tests as gold standard (used by no. 2), and microscopy for gold standard (used by no. 3).

Sample size & confidence intervals (CI)						
No.	**Sensitivity (%)**	**95 % CI**	**Specificity (%)**	**95 % CI**	**Total**	**Reference**
1.	90.7	78.9 - 96.5	98.7	91.8 - 99.9	129	(Haque et al., 2007)
2.	100	95.4 - 100	91.9	84.2 - 96.2	20	(Schuurman et al., 2007)
3.	98.1	92.5 - 99.7	70.6	52.3 - 84.3	138	(Verweij et al., 2003)

As a guide to the sample size verification, the accuracy figures from the study by Haque et al. (2007) (Table 3.3) was used because, for a gold standard, they used a composite reference standard (ELISA and PCR) in the design of the study as has been used in part in this study. So using Haque et al. (2007) measures as a reference guide, and assuming the expected sensitivity for this study to be ≥ 0.90 with the lower 95 % confidence limit not falling below 0.75, with 0.95 probability (power), the exact number of cases required for sensitivity estimation was 70 (Figure 3.3).

Figure 3.2: Sample sizes for studies (1).
The values are based on 0.95 power and the lower 95 % confidence limit with expected sensitivity or specificity ranging from 0.91-0.99 (source: Flahaut et al. (2005)).

Number of cases (or controls) for expected sensitivities (or specificities) ranging from 0.91 to 0.99

Expected sensitivity (or specificity)	Minimal acceptable lower confidence limit													
	0.85	0.86	0.87	0.88	0.89	0.9	0.91	0.92	0.93	0.94	0.95	0.96	0.97	0.98
0.91	319	438	666	1,127	2,443	9,309								
0.92	220	294	403	613	1,035	2,215	8,428							
0.93	166	203	273	372	549	934	1,992	7,512						
0.94	126	153	183	248	334	493	832	1,763	6,576					
0.95	93	109	137	169	217	298	434	729	1,524	5,626				
0.96	76	82	98	117	151	191	253	374	625	1,288	4,654			
0.97	59	63	79	85	105	129	158	224	309	519	1,036	3,643		
0.98	50	53	58	63	69	89	115	129	185	259	386	781	2,620	
0.99	50	50	50	51	56	61	68	77	109	127	181	261	521	1,567

The probability that the estimated 95% lower confidence limit is above the minimal acceptable value is 0.95.

Similarly, with specificity of 98.7 % (95 % CI: 91.8 to 99.9 %) from the same study (Haque et al. (2007)), assuming the expected specificity for this research study to be ≥ 0.98 with the lower 95 % confidence limit not falling below 0.89, with 0.95 probability (power), the exact number of cases required for specificity estimation was 69. The 170 samples (91 for sensitivity and 79 for specificity) used in this study therefore satisfied the sample size minimum requirement of 139 samples (70 for sensitivity and 69 for specificity) (Flahault et al., 2005) for the estimation of sensitivity ≥ 90 % and specificity ≥ 98 %.

Figure 3.3: Sample sizes for studies (2).
The values are based on 0.95 power and the lower 95 % confidence limit with expected sensitivity or specificity ranging from 0.60-0.95 (source: Flahaut et al. (2005)).

Number of cases (or controls) for expected sensitivities (or specificities) ranging from 0.60 to 0.95

Expected sensitivity (or specificity)	Minimal acceptable lower confidence limit								
	0.5	0.55	0.6	0.65	0.7	0.75	0.8	0.85	0.9
0.60	268	1,058							
0.65	119	262	1,018						
0.70	67	114	248	960					
0.75	42	62	107	230	869				
0.80	28	40	60	98	204	756			
0.85	18	26	33	52	85	176	624		
0.90	13	18	24	31	41	70	235	474	
0.95	11	12	14	16	24	34	50	93	298

The probability that the estimated 95% lower confidence limit is above the minimal acceptable value is 0.95.

With these verifications established, the set of results obtained from the testing of the 170 samples would therefore be adequate for the examination for statistical significance.

Statistical tests:

Before diagnostic sensitivities and specificities were calculated, McNemar statistics were performed to compare the index test with the CRS to establish

the extent of any differences in performance that there might be among the diagnostic tests. McNemar's test was used to analyse paired data obtained from the three index tests (OCP-M, Primerdesign Ltd. real-time PCR and Verweij real-time PCR) when used to test all the 170 samples in this study. This was done to find out any significant differences in performance among the three tests. The test data was analysed using a 2x2 table (Table 3.4):

Table 3.4: Two by two table in McNemar's test calculation.
It is used to test the difference between paired proportions or when the two classification factors are dependent.

Test	Negative	Positive	Total
Negative	A	B	(A+B)
Positive	C	D	(C+D)
Total	(A+C)	(B+D)	(A+B+C+D)

In McNemar's test, the null hypothesis assumes that the total rows are equal to the sum of columns, i.e. (A+B) = (A+C), and (C+D) = (B+D) (Table 3.4) and therefore there will be no significant difference in performance between the paired tests. This situation will occur in this study if the real-time PCR and the OCP-M performed at the same level testing the 170 stool samples. The alternative hypothesis assumes that the total number of rows is not equal to the total number of columns and therefore significant difference in performance exists between the paired tests. McNemar's test's calculated value is compared with the Chi-square table value. If the calculated value for McNemar's test value is greater than the table value, the null hypothesis is rejected. If, however, the calculated value is less than the table value, the null hypothesis is accepted. Statistical Product and Service Solutions (SPSS software) was used for the calculations. Cross tabulation statistics were also used to analyse the results data (Table 3.5). The sensitivity, specificity, and likelihood ratios were calculated using the following formula:

Table 3.5: Standard 2x2 table for the analysis of 170 test results.
Each of the three index tests provided 170 test results for analysis. A positive composite reference standard test result indicated the presence of giardiasis and a negative result indicated the absence of giardiasis.

Test	Composite reference standard		Total
	Positive	Negative	
Positive	a (TP)	b (FP)	(a+b)
Negative	c (FN)	d (TN)	(c+d)
Total	(a+c)	(b+d)	(a+b+c+d)

Key: TP= True Positive, TN= True Negative, FP= False Positive, FN= False Negative

Sensitivity = a/(a+c); Specificity = d/(b+d), positive likelihood ratio (LR+) = Sensitivity/ 1 – specificity, and negative likelihood ratio (LR-) = 1 – sensitivity/ specificity. Medcalc diagnostic test calculator was used to calculate the values with the accompanying confidence intervals.

3.2.3 Sequencing

MO BIO Laboratories UltraClean 15 DNA purification kit was used in an attempt to recover 62 bp *Giardia* DNA from TAE agarose gels. The kit can purify DNA size range 60 bp - 50 kb. The desired DNA band was cut from the agarose gel after electrophoresis and melted irreversibly in a chaotropic salt solution. The kit uses ULTRA BIND silica particles to bind DNA. The DNA/silica complex was pelleted by centrifugation and the pellet washed once before the concentrated DNA was collected in Tris buffer. The manufacturer's guidelines as provided in Appendix VI were followed in an attempt to obtain purified *Giardia* DNA for sequencing from the twenty samples with discrepant results from the Verweij real-time PCR (Table 3.8).

3.3 Results

Cross tabulation results:

The results of the 2x2 analysis for diagnostic sensitivities and specificities as well as positive and negative likelihood ratios are shown in Table 3.6 and 3.7.

Table 3.6: Diagnostic tests results.
The OCP-M and Verweij real-time PCR together formed an index test as well.

Test	No. of samples				Total
CRS	True positive		True negative		
EIA & RMT	91		79		170
Index	**True positive fraction**	**False positive fraction**	**True negative fraction**	**False negative fraction**	**Total**
OCP-M	76	3	76	15	170
Primerdesign	56	1	78	35	170
Verweij	85	20	59	6	170
OCP-M & Verweij	85	21	58	6	170

The Verweij real-time PCR, with detection limit < 5 cysts/ml, gave a sensitivity of 93.4 % (95 % CI: 86.2 to 97.5 %), which was the best among the three index tests (Table 3.7). The LOD of the Primerdesign Ltd. real-time PCR of ≤ 114 cysts/ml of stool produced a diagnostic sensitivity of 61.5 % (95 % CI: 50.8 to 71.6 %), whilst the LOD of the OCP-M of ≤ 14200 cysts/ml of stool gave a diagnostic sensitivity of 83.5 % (95 % CI: 74.3 to 90.5 %).

Table 3.7: Comparison of performance results of the index test.

Index tests	Sensitivity		Specificity		Likelihood ratios			
	%	95 % CI	%	95 % CI	LR+	95 % CI	LR-	95 % CI
OCP-M	83.5	74.3 to 90.5 %	96.2	89.3 to 99.2 %	22.0	7.2 to 67.0	0.17	0.11 to 0.27
Primerdesign	61.5	50.8 to 71.6 %	98.7	93.1 to 99.8 %	47.3	6.9 to 343.2	0.39	0.30 to 0.51
Verweij	93.4	86.2 to 97.5 %	74.7	63.6 to 83.8 %	3.7	2.5 to 5.4	0.09	0.04 to 0.19
OCP-M plus Verweij	93.4	86.2 to 97.5 %	73.4	62.3 to 82.7 %	3.51	2.4 to 5.1	0.09	0.04 to 0.20

LR+ = Positive likelihood ratio; LR- = Negative likelihood ratio; 95 % CI = 95% confidence interval.

The Verweij real-time PCR had the highest analytical as well as diagnostic sensitivity of < 5 cysts/ml and 93.4 % (95 % CI: 86.2 to 97.5 %) respectively compared with the ≤ 114 cysts/ml analytical sensitivity and 61.5 % (95 % CI: 50.8 to 71.6 %) diagnostic sensitivity of the Primerdesign Ltd. PCR. The gdh gene has been investigated for *Giardia* sp identification and has been found to be less efficient than that of the (SSU) rRNA gene (Boontanom, Siripattanapipong, Mungthin, Tan-ariya, & Leelayoova, 2010) and this study confirms that.

Table 3.8: Results of further investigation into false positive samples.
CPCR simulation of the Verweij real-time PCR was used to re-test 20 Verweij real-time positive samples classified as false positive by the CRS to confirm the presence of 62 bp amplicons. Cases 20 and 26 were also positive by microscopy but only case 20, with the lowest Ct, was independently confirmed to have 130 bp amplicons of *Giardia intestinalis*.

No.	Study case no.	CRS	CPCR		Verweij real-time PCR	
			Nested	Simulation of the Verweij PCR	Result	Ct
1	20	NEG	POS	POS	POS	18.66
2	80	NEG	NEG	POS	POS	33.41
3	26	NEG	NEG	POS	POS	33.99
4	89	NEG	NEG	POS	POS	34.31
5	74	NEG	NEG	POS	POS	34.82
6	73	NEG	NEG	POS	POS	35.04
7	21	NEG	NEG	POS	POS	35.66
8	34	NEG	NEG	POS	POS	36.80
9	51	NEG	NEG	POS	POS	37.13
10	103	NEG	NEG	POS	POS	38.77
11	12	NEG	NEG	POS	POS	39.08
12	104	NEG	NEG	POS	POS	39.99
13	11	NEG	NEG	POS	POS	37.14
14	101	NEG	NEG	POS	POS	38.50
15	44	NEG	NEG	NEG	POS	39.81
16	42	NEG	NEG	NEG	POS	36.42
17	57	NEG	NEG	NEG	POS	37.80
18	37	NEG	NEG	NEG	POS	38.13
19	67	NEG	NEG	NEG	POS	38.64
20	96	NEG	NEG	NEG	POS	38.78

CRS= Composite reference standard; CPCR = Conventional PCR; Ct = Cycle threshold; NEG = Negative; POS = Positive.

The OCP-M had a very low analytical sensitivity which is reflected in the naturally low diagnostic sensitivity of 83.5 % (95% CI: 74.3 to 90.5 %). This notwithstanding, microscopy is a very useful test in the sense that the presence of other parasites apart from *Giardia* can be detected.

The Verweij real-time PCR had about 20 apparent false positive test results because the positive results could not be confirmed by the CRS (Table 3.8) and these were recorded as false positives in the statistical calculations.

DNA extraction from gel for sequencing

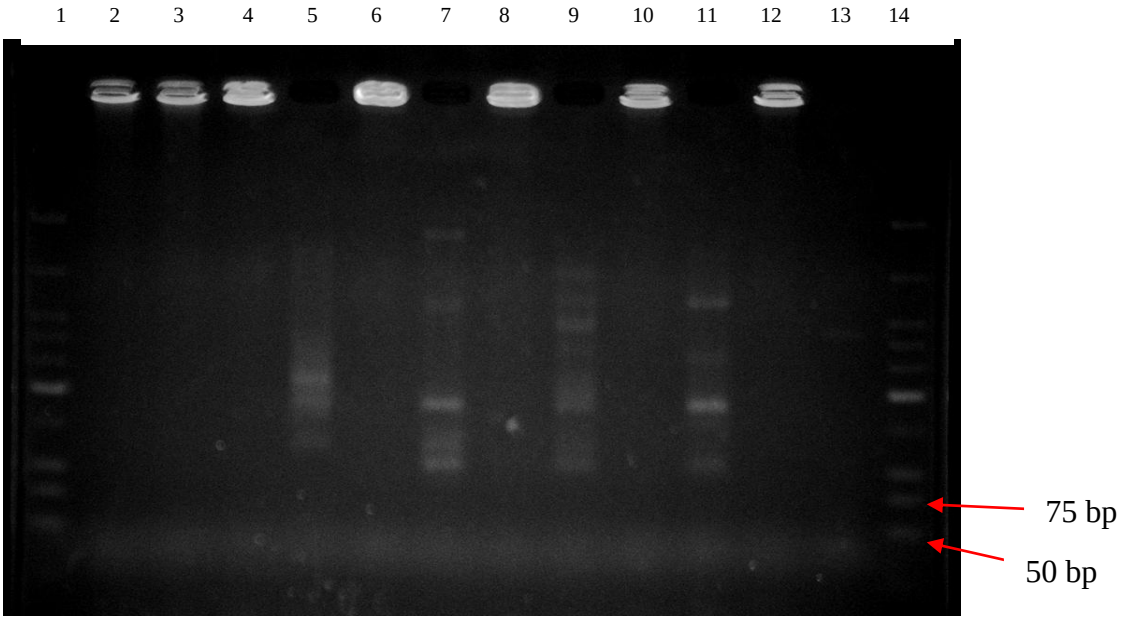


Figure 3.4: Agarose gel electrophoresis of the Verweij real-time PCR amplicons and the Conventional nested DNA products.
Samples were from the first five discrepant cases in Table 3.8 i.e. cases 20, 80, 26, 89, and 74. These cases were in lanes: 2, 3, 4, 6, 8, and 10 respectively and they failed to move out from the origin. Lanes: 5, 7, 9, and 11 were the conventional nested PCR products of cases 80, 26, 89, and 74 respectively. Lane 2 was duplicated in lane 3. Those that migrated in lanes 5, 7, 9, 11 failed to reveal the expected 62 bp bands for *Giardia intestinalis*.
Lanes 1 and 14 were Biolabs low molecular weight DNA LADDER and lanes 12 and 13 were positive control DNA template and non-template control (water) respectively. Twenty five µl products were run for 2 hours in a 3 % agaorse gel.

DNA sequencing, the process of determining the precise order of nucleotides within a DNA molecule, was planned for use to further investigate 20 false

positive samples. The sequencing was to be done at the London School of Hygiene and Tropical Medicine (LSHTM) where the facilities are based. However, all attempts to purify 62 bp *Giardia intestinalis* DNA, from the samples with discrepant results, for use in sequencing were unsuccessful from both the real-time PCR amplicons and from the conventional nested PCR assay (Figures 3.4, 3.5, 3.6). The 5 products that failed to move out from the origin were selected initially to begin the DNA extraction from gel because of their relatively low Ct. Even though all the twenty discrepant results showed high Cts, these five samples stood a better chance of showing bands on agarose gel. The DNA that failed to move from the starting point on the gel (Figure 3.4) were cut out and extracted using MO BIO Laboratories UltraClean 15 DNA purification kit.

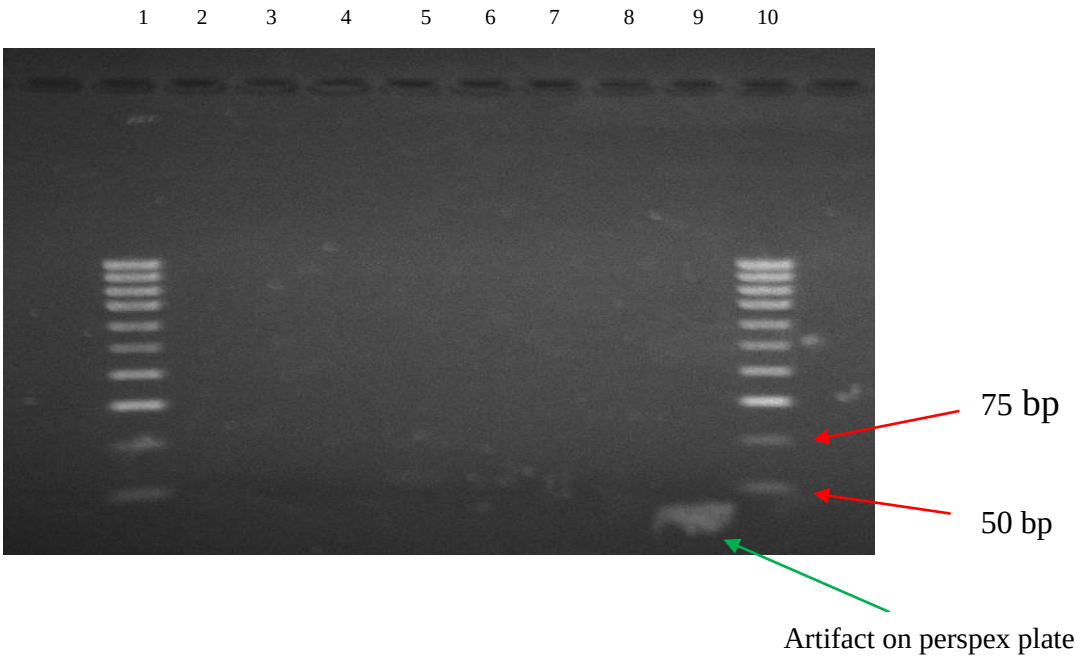


Figure 3.5: Agarose gel electrophoresis of selected Verweij real-time PCR discrepant results. These were cut out of the agarose gel in Figure 3.4. Lanes 1 and 10 were Biolabs low molecular weight DNA LADDER. Lanes 2 - 9 which included the five samples and the positive control from Figure 3.4 lane 12 showed no amplified products.

The electrophoresis was repeated on these extracted DNA samples and no *Giardia intestinalis* DNA was detected in any of the samples including the

positive control (Figure 3.5) when 2 µl of each extract was run on 3% agarose gel as a test run to show the presence of the required DNA bands which would have indicated a successful extraction and would have meant that 62 bp *Giardia intestinalis* DNA amplicons could be isolated and sequenced.

The pre-extraction procedures were not performed on the remaining 15 samples because of their relatively high Cts. They had even less chance of producing bands when samples with even less Cts values had failed to be extracted from the agarose gel. Figure 3.6 shows an example of the many fruitless attempts to get *Giardia intestinalis* 62 bp DNA for sequencing.

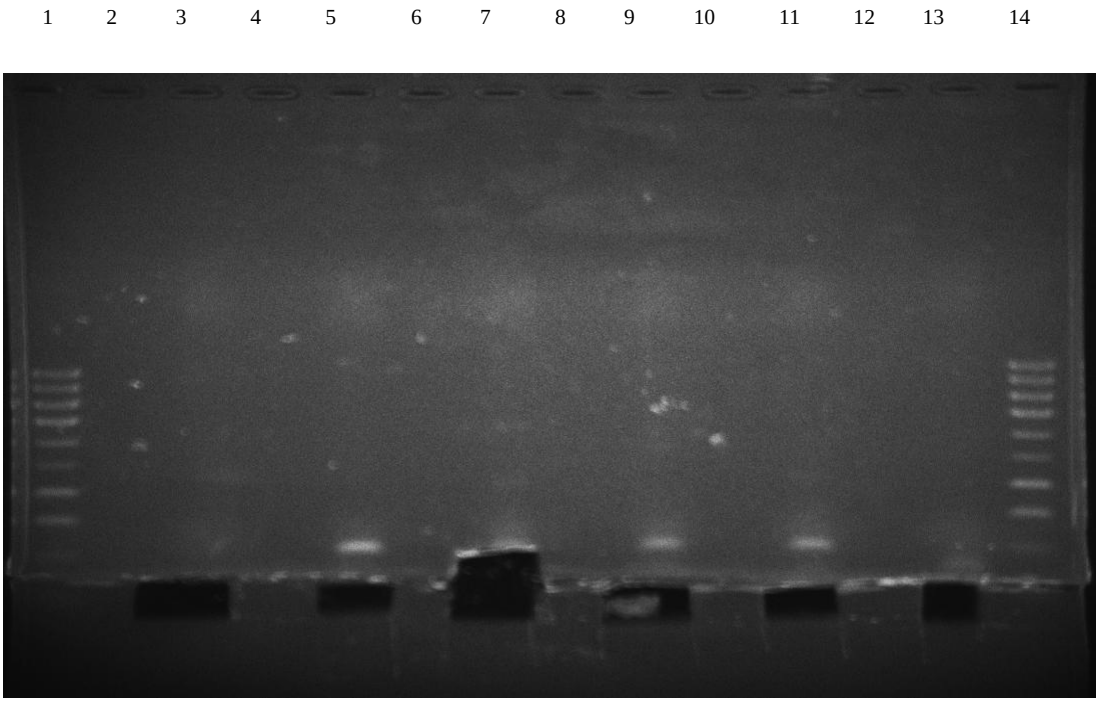


Figure 3.6: Electrophoresis gel showing the positions of excised DNA bands (holes in agarose gel). The positions were estimated to be that for the 62 bp amplicons for *Giardia intestinalis*.

A considerable amount of time was spent trying to resolve issues by varying the amounts of reagents and agarose gel concentrations used as well as the length of time gels were run (1.5 - 3 h). The exercise became too costly and in the end no 62 bp *Giardia intestinalis* DNA was isolated.

Alternative strategies that could have been explored, if time and resources had allowed obtaining a good yield of DNA for sequencing would be cloning of the

Giardia parasite. This procedure has been used in a multi-locus analysis of *Giardia intestinalis* (Wielinga, Ryan, Thompson, & Monis, 2011). It has also been explored in a novel method using cultures and faecal samples and no genetic difference was found between any of the clones and the parent isolates (Binz, Thompson, Meloni, & Lymbery, 1991). Also DNA could be pelleted and reconstituted with a small volume of buffer to increase it concentration (Personal communication, R. Chalmers 14/07/2013). Polyacrylamide gel electrophoresis was also suggested as it is capable of providing very high resolution of DNA molecules in the 10 - 3 kb size range.

Some researchers have used larger volumes of DNA solution (500 µl) with a measure of success (Calderaro et al., 2010) but the resources and time available for this study did not allow further experimentation in this area.

As a direct alternative to sequencing, restriction fragment length polymorphism (RFLP) together with PCR could also be used to further characterize *Giardia intestinalis*. RFLPs are detected and identified with the aid of DNA molecular markers when restriction digest are separated on agarose gel by electrophoresis. This procedure has been used to identify genotypes of *Giardia intestinalis* by targeting the gdh locus (Read et al., 2004). This is an area that could be explored in the future in situations where direct sequencing becomes a problem.

For this study, in the absence of direct sequencing, the presence of 62 bp fragments of *Giardia intestinalis* was investigated using conventional nested PCR. Sixty two bp fragments were the amplicon sizes produced by the SSU-rRNA primers of the Verweij real-time PCR. The decision was therefore taken to remove the probe from the Verweij real-time PCR and run the product, after amplification, on agarose gel to visualize 62 bp diagnostic bands that the Verweij real-time PCR primers were meant to amplify. This has been called CPCR simulation of the Verweij real-time PCR (section 2.2.6). Fourteen of the 20 samples appeared confirmed by the CPCR simulation of the real-time PCR to contain the target gene (62 bp *Giardia* DNA fragments) (Figures 3.4 and 3.5)

and the six samples in which the CPCR simulation failed to confirm the presence of 62 bp fragments were deemed more likely to be due to non specific amplification and less likely to be due to environmental contamination. The six samples had weak Ct reactions with values in the range of 37.8-39.8 (38-40) (Table 3.8) which suggested a minimal amount of target DNA (Real Time PCR Ct Values, n.d.). In this study a Ct of 35.7 was equivalent to 1.98 (~ 2) *Giardia* cysts and 36.8 was equivalent to 0.89 of a *Giardia* cyst (~ 1). Two of the 14 samples were positive by microscopy with Cts of 18.7 and 34.0 (Table 3.8). All 14 samples were retested with the nested CPCR and only the sample with Ct of 18.7 (no. 20) gave a positive result which indicated the identification of a 130 bp fragment of *Giardia intestinalis* (Figure 3.9).

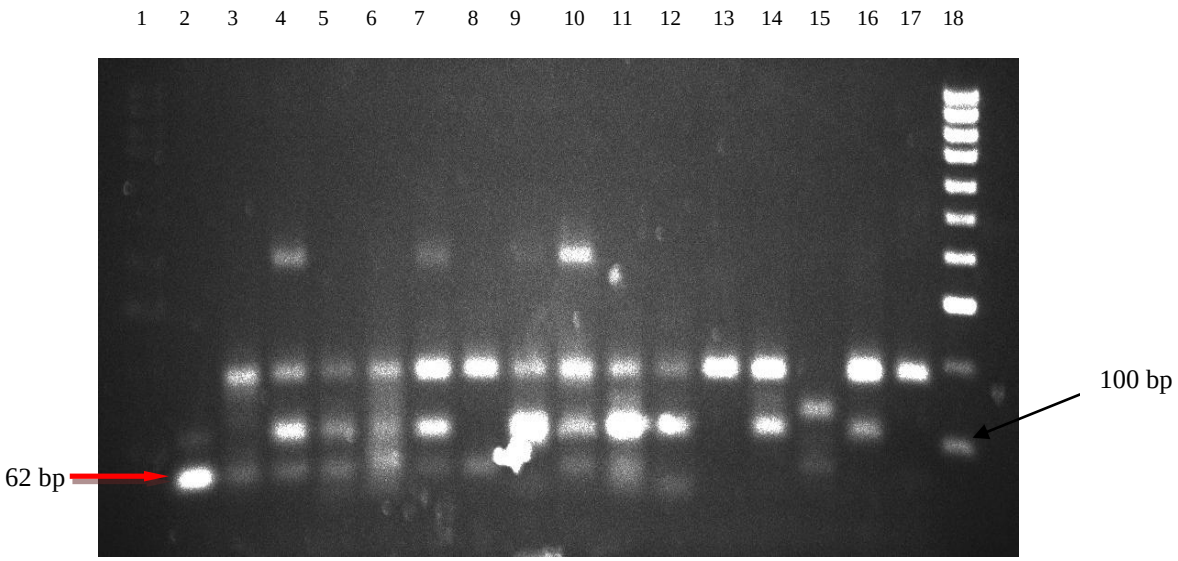


Figure 3.7: Conventional simulation of Verweij real-time PCR (1).
Agarose gel electrophoresis for *Giardia* was stained with Safeview. Lanes 1 and 18, 100 bp ladder (Gentaur); lanes 2-17 are sample numbers 20, 80, 26, 89, 74, 73, 21, 42, 34, 51, 11, 57, 37, 101, 67, 96 respectively. These 16 samples were all negative by the CRS. Positive and negative control runs are on gel in Figure 3.8. With the exception of lanes 9, 13, 14, 16, and 17 all the rest show diagnostic bands at 62 bp for *Giardia* (red arrow). That is eleven 62 bp positive samples and five negative samples.

Therefore, at least one result was independently verified. The Ct for the microscopy positive sample that failed to produce a positive result with the composite reference standard and the nested CPCR (study case no. 26) was 34 (33.99) (Table 3.8). The Verweij real-time PCR cut-off for a positive result was

40 and Cts of 30-37 are positive reactions indicative of moderate amount of target nucleic acid (Real Time PCR Ct Values, n.d.). The issue is that of sensitivity for the amount of starting DNA was below the detection level of both the CRS and nested CPCR.

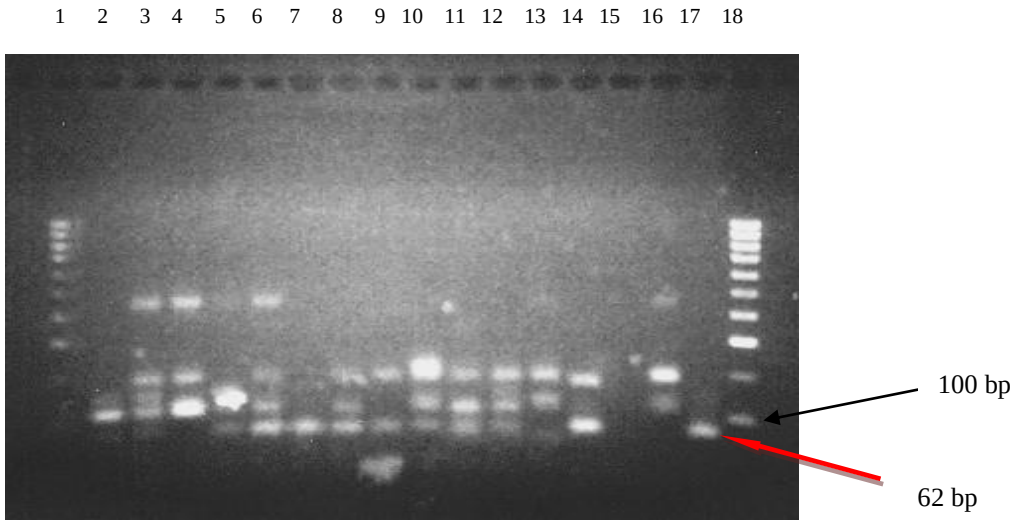


Figure 3.8: Conventional simulation of Verweij real-time PCR (2).
Agarose gel was stained with Safeview. Lanes 1 and 18 are 100 bp. ladder (Gentaur); lanes 2, 3, and 5 show diagnostic bands for 62 bp for *Giardia intestinalis* (red arrow)representing samples 103, 12, and 104 respectively; lane 4 is sample no. 44 and it is negative for 62 bp *Giardia intestinalis* amplicon. Lanes 14 and 17, positive control; lanes 15 and 16 are negative controls. So for the discrepant samples there are three positive samples and one negative sample on this gel (NB. Lanes 6-13 were known *Giardia intestinalis* positive samples run alongside the discrepant samples on this gel).

Table 3.9: Adjusted diagnostic accuracy figures for OCP-M and Verweij PCR.
Fourteen samples confirmed true positive were included in the CRS for the calculations.

Adjusted Diagnostic accuracy	Diagnostic test			
	OCP-M		Verweij real-time PCR	
	%	95 % CI	%	95 % CI
Sensitivity	75	65.55 to 82.97	94.3	87.97 to 97.86
Specificity	98.5	91.81 to 99.75	90.8	80.97 to 96.51
LR+	49.5	7.05 to 347.31	10.2	4.76 to 21.92
LR-	0.25	0.18 to 0.35	0.06	0.03 to 0.14

When the 14 samples that showed the 62 bp fragments were considered as true positive cases for the restricted comparison between the OCP-M and the Verweij real-time (Incorporation bias was avoided), the adjusted accuracy figures that ensued showed a much improved performance for the Verweij real-time PCR (Table 3.9). Specificity, for example rose up to 90.8 % (80.97 to 96.51 %) from 74.7 % (63.6 to 83.8 %).

A combination of tests was also investigated by combining OCP-M and the Verweij real-time PCR to form an index test to see whether the combined effect will maximize the sensitivity of the *Giardia* assay. The result showed no improvement in sensitivity which remained at the same level of 93.4 % (Table 3.7). The specificity however was reduced as a result of the combination and fell from 74.7 % (95 % CI: 63.6 to 83.8 %) down to 73.4 % (95 % CI: 62.3 to 82.7 %).

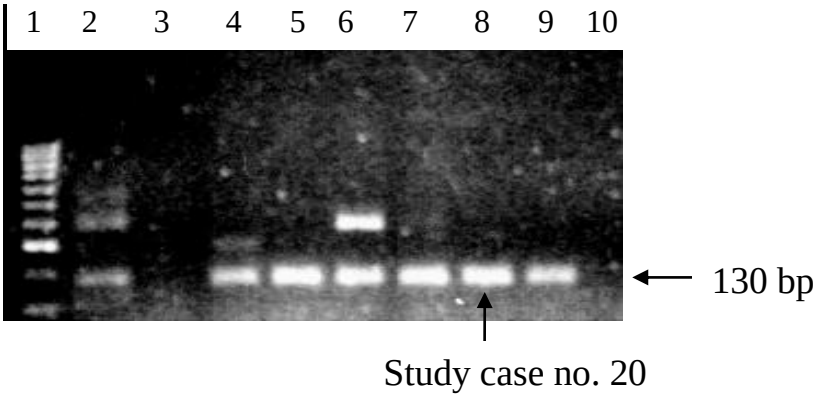


Figure 3.9: Conventional nested PCR of case no. 20.
This run included other *Giardia* positive samples. Agarose gel was stained with Safeview.Lane 8 is case no. 20 showing 130 bp amplicon of *Giardia intestinalis*. Lane 1 is 100 bp ladder (Gentaur). Lane 3 negative control, and lanes 2, 4-7, and 9 are positive controls. Lane 10 is study case no. 12, Ct 39.08 showed negative and so did all the rest of the 14 samples apart from case no. 20 in lane 8.

McNemar's test results:

For all three of the paired tests (i.e. Verweij real-time PCR vs. OCP-M, Verweij real-time PCR vs. Primerdesign Ltd. PCR, and OCP-M vs.

Primerdesign Ltd. PCR), p-values were < 0.05, which was statistically significant (Tables 3.9-3.11). Therefore the null hypothesis that said there was no difference in the performance of the three tests was rejected. The index tests produced different sets of results on the same 170 samples and the p-values (< 0.05 in each case) (Table 3.10, Table 3.11, Table 3.12) indicated that there were significant differences in performance among the tests and this warranted further investigations.

Table 3.10: McNemar's test result - OCP-M vs Verweij assay.

Test		OCP-M		Total	p-value
		-	+		
Verweij real-time PCR	-	64	1	65	< 0.05
	+	27	78	105	
Total		91	79	170	

Table 3.11: McNemar's test: OCP-M vs Primerdesign Ltd. assay.

Test		OCP-M		Total	p-value
		-	+		
Primerdesign Ltd. real-time PCR	-	87	26	113	< 0.05
	+	4	53	57	
Total		91	79	170	

Table 3.12: McNemar's test: Verweij vs Primerdesign Ltd. assay.

Test		Primerdesign Ltd. real-time PCR		Total	p-value
		-	+		
Verweij real-time PCR	-	65	0	65	< 0.05
	+	48	57	105	
Total		113	57	170	

3.4 Discussion

The aim of this study was to determine the diagnostic accuracy of real-time PCR methodology as a frontline test for the laboratory diagnosis of giardiasis. In any diagnostic accuracy study, the performance of the gold standard is crucial and because there is no established gold standard for the investigation of *Giardia intestinalis* (Vesy & Peterson, 1999), a composite reference standard (CRS) of EIA and RMT was adopted together with two conventional PCR (CPCR) assays to act as confirmatory tests for discrepant results (Table 3.8). The CRS in this study had an estimated detection limit of ≤ 2840 cysts/ml compared with the ≤ 114 cysts/ml detection limit of the Primerdesign Ltd. real-time PCR and < 5 cysts/ml of the Verweij real-time PCR. Using both manual and automated DNA extraction methods and different reaction volumes but the same Verweij real-time PCR primers and probes, Calderaro et al. (2010) reported detection limit of 2000 cysts/g of stool for the Verweij real-time PCR. This corresponded to a theoretical value of 2 cysts/ reaction and again emphasised the analytical superiority of the Verweij real-time PCR over the CRS.

The CRS detection limit showed that *Giardia* parasites would be detected by the two index real-time PCR assays that the CRS failed to detect. This situation partly accounted for the highest number of false positive test results (about 20 of them) that the Verweij real-time PCR produced and had to be investigated further first with CPCR simulation of the Verweij real-time PCR and then with a nested CPCR (Table 3.8). This composite reference standard approach differed from previous investigators (Elsafi et al., 2013; Schuurman et al., 2007) who used experimental designs that utilized combinations of index tests as extended reference test in the absence of a gold standard. The approach in this study was however similar to that used by Calderaro et al. (2010) to get around the problem of non-availability of a gold standard for *Giardia* investigations. They used a composite reference standard of microscopy (OCP-

M and direct immuno-fluorescence) and ImmunoCard STAT! enzyme immunoassay assay. They reported a total of 26 false positive results out of a total number of 602 true negative samples tested resulting in a percentage false positive rate of 4.3 % [(26/602) x 100]. Using the same set of primers and probe that Calderaro et al. (2010) used, the percentage false positive rate in this study was 25.3 % [(20/79) x 100]. The difference could be due to the fact that the researchers used microscopy for their gold standard and with a sensitivity of 86.7%, selected samples with reasonable amount of *Giardia* parasites that could not be easily missed by the Verweij real-time PCR. In other words fewer false positives (4.3 %) and no false negative (0 %) were reported which produced a calculated specificity of 95.7 % and a sensivity of 100 %. They however reported a sensitivity of 100 % as well when the 26 false positives were shown to have the 62 bp fragments. It also showed that the non-microscope based CRS in this study performed less than the ideal gold standard and therefore incorporated more positive cases with lesser amount of *Giardia* parasite as true negatives that were detected by the Verweij real-time PCR hence the higher percentage rate of 25.3 % for false positives and 6.6 % for false negatives resulting in sensitivity of 93.4 % and specificity of 74.7 %.

The strength of the CRS in this study was in the EIA component which was analytically more sensitive than the two real-time PCRs in detecting *Giardia* trophozoites. This suggests that in situations where *Giardia* trophozoites are encountered more than cysts (e.g. as in duodenal aspirates), the EIA will be a more useful test to use. *Giardia* cysts are however found in stools more than trophozoites and the use of the CRS was still limited in spite of the high analytical sensitivity it showed. The CRS however avoided incorporation bias with the index tests which otherwise could have inflated accuracy measures like sensitivity and specificity. This was certainly the case when Calderaro et al. (2010) included their 26 false positive samples with the true positives after showing that they were true *Giardia intestinalis* DNA using conventional PCR

followed by sequencing and obtained a sensitivity of 100% and specificity of 100% (Calderaro et al., 2010). Without the inclusion of the 26 cases as true positives, the specificity would have been 95.7%.

This appears to be the first time an independent and a comprehensive evaluation of *Giardia intestinalis* diagnostic tests has been performed in a hospital laboratory setting in the UK. The gene target for the Primerdesign Ltd. PCR was the gdh gene and that for the Verweij real-time PCR was the (SSU) rRNA gene (Verweij et al., 2004; Verweij et al., 2003). The SSU-rRNA sequence has been found to be more conserved and could be the explanation for why primers amplifying the (SSU) rRNA gene worked better (Nantavisai et al., 2007).

The nested conventional PCR used as one of two confirmatory tests in this study confirmed one out of 14 samples tested for 130 bp amplicons of *Giardia intestinalis*. The nested PCR did not detect *Giardia* DNA in 13 of these 14 samples even though the first confirmatory test (i.e. CPCR non-probe based simulation of the Verweij real-time PCR) gave positive results (Table 3.8) and diagnostic bands of 62 bp fragments were seen on electrophoresis gels (Figure 3.7 and Figure 3.8). It is apparent that the nested CPCR detection limit was higher than that of the Verweij real time PCR and so the samples with Cts in the range of 33.4-40, indicating moderate to minimal copies of DNA target in the amount of specimen extracted, were not detected (Real Time PCR Ct Values, n.d.) (Table 3.8).

No bands were observed when the real-time PCR amplification products for the discrepant cases were run on agarose gel (Figure 3.5). Calderaro et al. (2011) reported a similar problem in their study with the explanation that the problem could be related to the poor amount of DNA amplicon in the real-time PCR product. There is the possibility of DNA quality issues as well for in this study the real-time PCR products did not migrate from the gel wells under electrical charge (electrophoresis) (Figure 3.4) and when the DNA were excised

from the gel and extracted, no bands were detected after repeating the electrophoresis. The DNA amplicons may have been denatured by the real-time PCR procedure. Further investigation into this was however not undertaken because of time and money constraints. Calderaro et al. (2011) managed to sequence three out of six samples only after using a larger amount of DNA (500 µl) for their conventional PCR which yielded enough DNA amplicons for sequencing. In this study the total amount of the original DNA extract was only 100 µl and this hampered the amount of further or repeated testing that could be done.

There was only one apparent PCR amplification failure where cysts were seen on microscopy but the Verweij real-time PCR failed to give a positive result even though the GFP extraction control was positive. The problem was a sampling issue for the portion of the stool used did not have *Giardia intestinalis* cysts due to the unequal distribution of cysts in the sample. The problem was resolved when the sample (study case no. 142) was re-extracted and the PCR repeated.

The Primerdesign Ltd. real-time PCR sensitivity was 61.5 % (95 % CI: 50.8 to 71.6 %) with an efficiency of 100 % (the slope = -3.342) using the same environmental mastermix as the Verweij real-time PCR. Generally an efficiency between 90-110 % is what is considered acceptable (Real-time PCR: Understanding Ct, 2011). The low diagnostic sensitivity for the Primerdesign Ltd. assay was due to the relatively high number of false negatives (FN) recorded, 35 in total. Because sensitivity relates inversely to the number of FNs i.e. Sensitivity = true positive/(true positive + false negative), any increase in the false negative samples will adversely affect sensitivity. The diagnostic specificity for the Primerdesign Ltd. real-time PCR was 98.7 % (95 % CI: 93.1 to 99.8 %). This means that a test result that is positive rules in giardiasis (SpPIN: a highly Specific test if Positive, rules IN disease). The Primerdesign Ltd. real-time PCR was not robust enough because *Giardia* DNA at lower

concentrations was not detected (Table 2.12, Tubes 6 and 7). The real-time PCR will therefore need optimization to overcome PCR inhibitors in faecal samples in order to perform efficiently on clinical samples. Until then its usage will be in the research laboratory delineating *Giardia intestinalis* complex into Assemblages A and B.

The Verweij real time PCR appears to be the most useful test for consideration as a frontline test for the laboratory diagnosis of giardiasis with a sensitivity of 93.4 % (95 % CI: 86.2 to 97.5 %), reaction efficiency of 96 % (the slope = -3.414) and R^2 = 99. The relatively reduced diagnostic specificity for the Verweij real-time PCR, 74.7 % (95 % CI: 63.6 to 83.8 %), was due to the 20 samples classified as false positive (FP) by the composite reference standard (Table 3.8). Because Specificity = true negative/(true negative + false positive), any increase in the number of false positive samples will lower the specificity figure. Upon further investigation, only 6 out of the 20 samples appeared to be genuinely false positive due perhaps to non-specific amplification rather than contamination. The clinical relevance of this assay is seen in the fact that, in this project, had it not been for the use of the Verweij real-time PCR assay, 10 % of giardiasis positive cases would have been diagnosed as not having *Giardia intestinalis* by using the OCP-M. OCP-M, on the otherhand, with a sensitivity of 83.5 %, failed to detect 16.5 % of true giardiasis cases in this study. Similarly, with a sensitivity of 93.4 %, the Verweij real-time PCR failed to detect 6.6 % *Giardia* positive cases. The Verweij real-time PCR therefore diagnosed approximately 10 % more giardiasis cases than the OCP-M. Also, the Verweij real-time PCR's robustness and excellent performance meant that 70 % (14/20) of the discrepant cases were correctly identified as cases of giardiasis. The OCP-M correctly identified only 10 % (2/20) (Table 3.8) and when these 14 samples are accounted for as true positive samples, the specificity and the likelihood ratios showed a remarkable improvement in performance of the Verweij real-time PCR over the OCP-M (Figure 3.9). The real-time PCR has

become an appealing alternative to conventional methods for diagnosing infectious diseases (Espy et al., 2006) and has also made the requirement to submit three stools for *Giardia* clearance non critical. A single stool sample, suitably timed post treatment, may be all that is required for the test of cure in the absence of other pathogenic intestinal parasites. With an enclosed system to minimise contamination risk coupled with the ease of performance, the real-time PCR technology has become an appealing alternative to conventional methods for diagnosing infectious diseases (Espy et al., 2006).

The specificity of the Verweij real-time PCR in this study will rise with the reduction in the number of false positive samples but in order to avoid the risk of verification bias, the specificity was not recalculated for this diagnostic accuracy study and was left at 74.7 % (95 % CI: 63.6 to 83.8 %) which was even better than what the original researchers, Verweij et al. (2003), had (i.e. a specificity of 70.6 % (95 % CI: 52.5 to 84.9 %)). However, as a potential diagnostic assay to replace the OCP-M, the Verweij Real-time PCR showed a relatively better improvement over the OCP-M when both were compared in an isolated case using the adjusted accuracy figures when the 14 confirmed true positives were noted in the CRS as true positive cases (Table 3.9). The specificity for the Verweij real-time PCR went up to 90.8 % (80.97 to 96.51 %) and the OCP-M sensitivity fell to 75 % (65.55 to 82.97).

Another issue that affect diagnostic specificity of PCR is “viability”. The PCR assay may detect *Giardia* DNA fragments from dead parasites incapable of reproducing or causing disease. The test has not lost diagnostic specificity but gives results that will need clinical information about the patient for treatment purposes (Saah & Hoover, 1998).

A highly sensitive test like the Verweij real-time PCR with very low LOD is vulnerable to contamination and in the light of this, the cut-off Ct value for a positive test result has been set at 40 and any Ct value above this is reported as

negative provided the GFP internal control Ct is within the acceptable range as previously mentioned.

Positive predictive values (PPV) and negative predictive values (NPV) depend on disease prevalence and in diagnostic accuracy studies do not offer information that can be generalized. The disease prevalence in this study is about 53.5 % and this figure can easily change with a change in the positive samples to negative samples ratio in the sample size. Because the prevalence in the clinical population being examined has to be identical to the prevalence in the study population from which the predictive values are derived, the usefulness of predictive values in diagnostic accuracy studies for making clinical decisions is always going to be limited (Cook, Cleland, & Huijbregts, 2007).

Likelihood ratios are clinically more useful for they provide information on how many times more (or less) likely patients with, e.g. giardiasis, are to have a particular result than patients without giardiasis. A positive likelihood ratio greater than one means that a positive test result is more likely to occur in people with giardiasis than in people without giardiasis (It increases certainty about a positive result). A positive likelihood ratio less than one means that a positive test result is less likely to occur in people with giardiasis compared to people without. A negative likelihood ratio, when greater than one, means that a negative test for giardiasis is more likely to occur in people with the disease than in people without it. When negative likelihood ratio is less than one, a negative test is less likely to occur in people with giardiasis compared with people without giardiasis (Akobeng, 2007). The greater than one the likelihood ratio is, the stronger the evidence of disease association. Conversely, the less than one the likelihood ratio is, the stronger the association with the absence of disease (Deeks & Altman, 2004). Likelihood ratios above 10 and below 0.1 are considered to provide strong evidence to rule in or rule out diagnoses respectively in most circumstances (Jaeschke, Guyatt, & Lijmer, 2002). The

OCP-M and Primerdesign Ltd. PCR had positive likelihood ratios (PLR) above 10 indicating a strong likelihood of disease if test results are positive, however, the large width of the confidence intervals i.e. 95 % CI: 22.0 (7.2 to 67) for OCP-M and 95 % CI: 47.3 (6.9 to 343.2) for the Primerdesign Ltd. real-time PCR make these likelihood ratios less precise. Three things affect the width of confidence intervals. They are sample size, level of confidence, and variability (standard deviation)(Jones et al., 2003). Given that the sample size (170) and the confidence interval level (95 % CI) were fixed in this study, the increased width of the confidence intervals for the OCP-M and the Primerdesign Ltd. real-time PCR could be attributed to the increased variability in the spread of the data generated by these two assays. The data differed significantly and to a greater degree more than the Verweij real-time PCR results, when all were compared with the CRS. The OCP-M and the Primerdesign Ltd. real-time PCR negative likelihood ratios (NLR) were 0.17 and 0.39 respectively but when compared with that of the Verweij real-time PCR of 0.09, are not small enough to rule out giardiasis with confidence. They indicate low likelihood of disease according to Cook et al. (2007) diagnostic value guidelines. In this study, the Verweij real-time PCR gave a PLR of less than 10 (i.e. 3.7) and showed a small increase in the likelihood of disease. The assay with its high sensitivity of 93.4 % gave 0.09 NLR which provided a strong evidence to rule out giardiasis with confidence (NLR = ((1-sensitivity)/Specificity). The PLR value of the Verweij assay was affected by its modest specificity of 74.7 % that produced a PLR value which was less than 10 (PLR = Sensitivity/(1-specificity)). The PLR value however rose up to 10.2 % (4.76 to 21.92) when the 14 confirmed true positive samples were included in the CRS as true positives. The NLR also improved with a value of 0.06 (0.03 to 0.14) (Table 3.9). These adjusted figures are mentioned here only in the context of an isolated comparison of the Verweij real-time PCR with the OCP-M and not in relation with the other diagnostic tests.

Sensitivities and specificities describe how a condition is associated with a particular set of results. They do not predict the risk of having the condition or abnormality which is of particular interest in clinical practice. Because of the reliance of predictive values on the prevalence of an abnormality, likelihood ratios are more acceptable in diagnostic accuracy studies and are used to calculate the likelihood of the abnormality.

Of the three index tests evaluated, the OCP-M method with a sensitivity of 83.5 % (95% CI: 74.3 to 90.5 %), failed to detect 16.5 % of *Giardia* parasites. The Primerdesign Ltd. PCR is not as sensitive as the Verweij real-time PCR and requires optimisation. The Verweij real-time PCR was compared with a combination of it and OCP-M and there was no improvement in diagnostic sensitivity which remained at 93.4 %. The OCP-M, however, detects other parasites that singleplex real-time PCR will not find.

The storage of stool samples was investigated and the results indicate that for a period of three months, storing stools at 4 ^{o}C with IMS was to be preferred above all the conditions tested. Storage at RT with IMS was also satisfactory but not as good as 4 ^{o}C with IMS. At -20 ^{o}C freezer condition, there was no significant benefit whether IMS was added or not. Either way, the result was not as good as storing at fridge temperature (4-6 ^{o}C) with IMS. IMS as a common replacement for absolute ethanol is readily available and the finding that IMS preserved stool samples stored at 4-6 ^{o}C gave the best storage condition prior to the extraction of DNA is useful to discover. Some laboratories usually avoid room temperature storage by keeping untreated stools at 4-6 ^{o}C and sometimes at -20 ^{o}C. The problem with -20 ^{o}C is that as the samples get taken out from the freezer and put in the post, they thaw out and as a result cysts rupture and release their DNA contents which then become subjected to degradation as has been discussed previously. IMS can be added to stool and stored in the fridge to slow down any deterioration of DNA during transportation.

Chapter 4: The influence of different storage conditions on *Giardia intestinalis* DNA detection

4.1 Introduction

Hospital for Tropical Diseases (HTD) receives stool samples from general practitioners and district general hospitals mainly in England but on occasion from any part of the UK. How the stool samples are stored is important for the success of subsequent molecular analyses. In a study conducted by Palayo et al. (2008) when they studied *Giardia* genotypes among Cuban children, they reported as unfortunate their inability to obtain genotyping data on 75 out of 95 *Giardia*-positive samples as only 20 (21 %) were successfully genotyped. They hypothesised that the low success rate was due to the storage conditions, -20 ^{o}C in 70 % ethanol. The degraded DNA was thought to have led to the false negative findings. They were, however, not able to explore the effect of storage on samples meant for molecular amplification studies.

Many factors can affect the integrity of DNA in a specimen, e.g. preservative treatment, age of specimen, and environmental factors like storage temperature (Lindahl, 1993). The degradation of the DNA could be hydrolytic or oxidative and the sites of the DNA molecule particularly vulnerable to these attacks are depicted in Figure 4.1. The consequence of these attacks is that the DNA molecule loses its integrity and therefore becomes undetectable. Denaturants like excessive heat and hydrolytic agents can open up or disrupt the duplex nature of DNA and cause accelerated damage. Also, chemical modification of

the nucleotide sequence can occur, resulting in alteration of how the DNA reacts chemically. Biological specimens can undergo autolysis and preservative treatment of these samples is aimed at halting that (Brown, 1999).

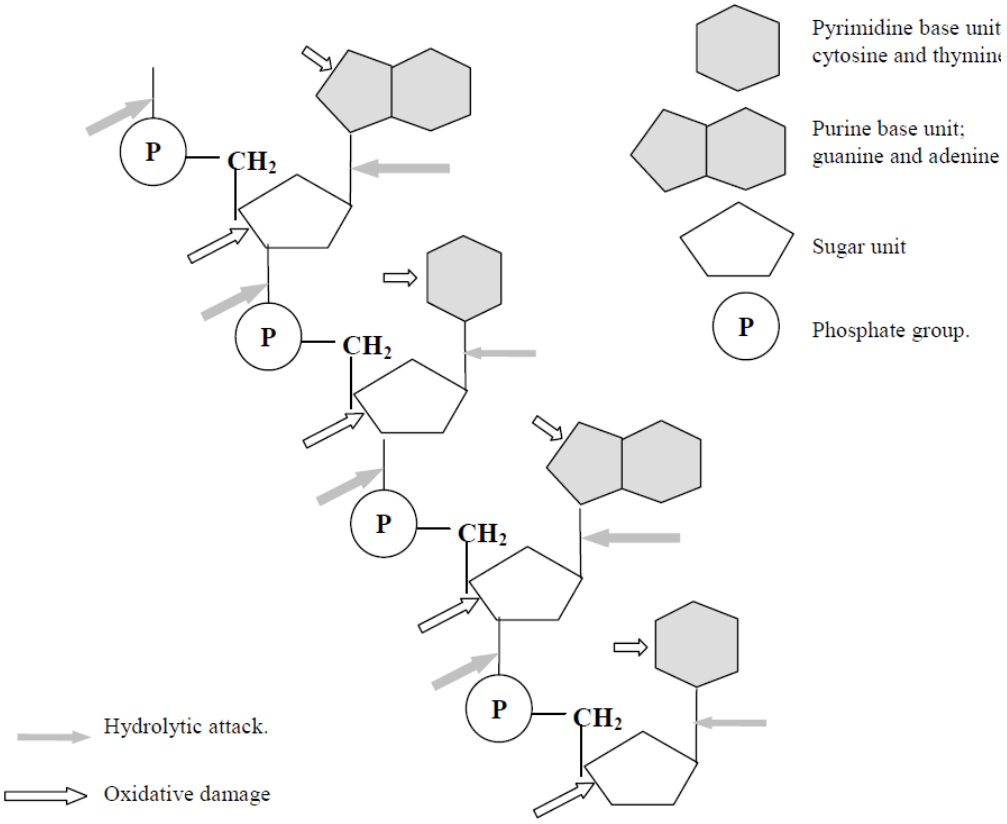


Figure 4.1: Target sites for DNA decay (after Lindahl, 1993).

Use of primary alcohol, ethanol, as a preservative causes precipitation of proteins and also replaces water molecules in a specimen (Pearse, 1980; Stoddart, 1989). Industrial methylated spirit (IMS) should have a similar effect on specimen preservation as ethanol for it is made up of approximately 95 % ethanol and 4 % methanol. The addition of the methanol makes IMS exempt from the customs duty that ethanol attracts, hence reducing its cost. IMS is therefore much more affordable than ethanol resulting in its wide scale use as a biological preservative (Carter, 2003). This chapter presents the investigation of the effect of IMS at room temperature, ordinary fridge temperature (4-6 ^{o}C), and

freezer temperature (-20 ^{o}C) on the extraction of *Giardia* DNA from stool samples. The facilities for these storage conditions are available in the Department of Clinical Parasitology HTD and they are also expected to be available in any standard district hospital laboratory. Stool specimens can be preserved in potassium dichromate 2.5 % (1:1 dilution) or in absolute ethanol (1:1 dilution) for molecular analysis to be performed (Wilke & Robertson, 2009). However, IMS was selected for investigation because it is a stock consumable in most laboratories and would be cost effective. The Verweij real-time PCR assay having proved to be the most sensitive of the tests compared was used to test samples stored under different conditions in order to provide answers for the following three questions:

1. Has IMS any effect on storage? i.e. is there any difference in the paired ΔCts of samples stored in IMS vs. no-IMS?
2. Has temperature any effect on storage? i.e. is there any difference in the ΔCts when the different temperatures are compared?
3. Which conditions emerge as the best for sample storage pending DNA extraction?

4.2 Methods

The Verweij real-time PCR was used for this part of the research project. The methodology has been previously described.

Samples and storage conditions

An OCP-M *Giardia* positive stool was used in this study. IMS was added to a portion of the stool sample (1:1) and was vortexed to mix. The stool was divided into 12 replicate pea-size samples (0.2-0.3 g) and kept in 1.5 ml Sarstedt tubes. The other portion of the stool was used untreated (i.e. no IMS or any other additive was added) and was also divided into12 replicate pea-size (0.2-0.3 g) samples and kept in 1.5 ml Sarstedt tubes. A total of 24 samples were prepared for use in this study and these were made up as IMS and no-IMS to

provide 12 paired stool samples. The stool was tested to provide baseline estimation for the Ct value of *Giardia* DNA. This step is equivalent to what Wilke and Robinson (2009) did when they studied the preservation of *Giardia* cysts in stool samples for subsequent PCR analysis. They assayed seven replicate aliquots per sample at each sampling point and then found the average of each seven (Wilke & Robertson, 2009). In this study, each storage temperature was assayed four times within a period of three months (Table 4.1). Pairs of samples were taken out on four occasions at approximately 0.7-0.8 month intervals and assayed using the Verweij real-time PCR previously described.

Table 4.1: Twenty-four samples and storage conditions.
Sample replicates represent the four occasions in the 3-month period when samples were assayed; each temperature had 4 replicates each for IMS and no-IMS conditions.

Storage condition for 3 months	**Sample replicates**			
No-IMS	**1**	**2**	**3**	**4**
Room temperature	√	√	√	√
Fridge (4–6 ^{o}C)	√	√	√	√
Freezer (-20 ^{o}C)	√	√	√	√
IMS				
Room temperature	√	√	√	√
Fridge (4–6 ^{o}C)	√	√	√	√
Freezer (-20 ^{o}C)	√	√	√	√

4.2.1 Statistical analysis

The Friedman test was used to calculate the effect of temperature on the amount of DNA yield expressed as delta Cts (ΔCts) from *Giardia intestinalis* positive stool samples following three months in storage. It tests for differences in related data and gives the overall difference and does not pinpoint which groups in particular differ from each other (Laerd Statistics: SPSS Tutorials, 2012). To

pinpoint which groups in particular differ, *a post-hoc* test was performed using Wilcoxon matched-pairs signed-rank test with the pairs matched for a particular sample at a particular sampling point (Laerd Statistics: SPSS Tutorials, 2012). To test for the effect of IMS on the amount of DNA yield from *Giardia* positive stool samples, the Wilcoxon rank-sum test instead was used. It is based on repeated-measures designs and it is used to determine whether participants changed significantly across occasions (or conditions) with two independent groups involved. This test is similar to the Mann-Whitney test and is limited to nominal variables with only two values. The Wilcoxon matched-pairs signed-rank test, the Wilcoxon rank-sum test, and the Friedman test rearranged the original dataset into ascending or descending order by a system of numbering called ranking. The mean of the ranks was used to show differences across groups.

4.3 Results

Table 4.2: Results of samples stored at different storage conditions.
These samples were assayed on 4 occasions from the end of the first month to the end of the third month. Ctn- Cto = ΔCt. Where Cto was the starting cycle threshold before the samples went into storage and Ctn represents the subsequent assay results.

Storage for 3 months	**Cycle threshold values (Ct) of samples tested on 4 different days in 3 months**								**Total ΔCt (ΣΔCt)**	**Mean Δ Ct**
	1		2		3		4			
No IMS, Cto = 23.2	Ct_n	ΔCt	Ct_n	ΔCt	Ct_n	ΔCt	Ct_n	ΔCt		
Room temperature	32.7	9.5	32.3	9.1	32.7	9.5	33.1	9.9	38	9.5
Fridge (4–6 ^oC)	26.7	3.5	27.7	4.5	26.8	3.6	31.4	8.2	19.8	5.0
Freezer(-20 ^oC)	26.3	3.1	26.1	2.9	23.6	0.4	27.3	4.1	10.5	2.6
IMS, Cto = 21.5	1		2		3		4		ΣΔCt	Mean Δ Ct
Room temperature	23.6	2.1	24.7	3.2	25.2	3.7	24.8	3.3	12.3	3.1
Fridge (4–6 ^oC)	23.3	1.8	23.0	1.5	22.2	0.7	23.7	2.2	6.2	1.6
Freezer (-20 ^oC)	27.0	5.5	22.9	1.4	22.6	1.1	24.3	2.8	10.8	2.7

The analysis has been presented under the two sub-headings of: Effect of temperature and Effect of IMS. There was an increase in Cts for all the samples over the 3-month period under all the different conditions. This is represented by positive delta Cts (ΔCts) in Table 4.2. This showed DNA degradation in storage and the most affected condition was storage at room temperature without the addition of IMS. It had the highest mean delta Ct (ΔCt) of 9.5. Analysis of how temperature and IMS affected the detection of *Giardia* cysts DNA now follows.

4.3.1 Effect of temperature

The calculated Friedman's test p-values for both the IMS treated and untreated samples shown in Table 4.2 were χ^2 (2, N = 4) = 4.500, p = 0.105 and χ^2 (2, N = 4) = 8.000, p = 0.018 respectively. In each case, the dependent variable was "delta Cts" and the independent variable was "temperature" which consisted of three categories: room temperature, fridge (4-6 ^{o}C), and freezer (-20 ^{o}C). With the IMS treated samples, there was no statistically significant difference in the DNA yield and therefore pairwise comparisons were not performed. Stool samples for molecular studies can therefore be treated with IMS and kept or transported at any of the three temperatures within 3 months. There was, however, a significant difference in the medians of the Cts for the untreated stool samples, χ^2. Because the overall test was significant, pairwise comparisons among the three groups was completed by analyzing the data with Wilcoxon signed-rank tests in *a post-hoc* analysis. Type I error (i.e. calling a result significant when it is not) across tests was controlled by using the Bonferroni approach. The results of these tests indicated no significant difference among the three pairs of room temperature vs. fridge (z = - 1.826, p = 0. 0.125), room temperature vs. freezer (z = - 1.826, p = 0.125), and fridge vs. freezer (- 1.826, p = 0. 0.125) conditions. The a priori alpha level (0.05) divided by the number of tests (i.e. 3) gave p = 0.017 (Bonferroni adjustment). The p-

value of 0.017 was therefore used to judge the level of significance in the *post-hoc* analysis. After the adjustment, it turned out that none of the results were significant (Table 4.3). Thus, the spuriously significant difference detected by the Friedman's test among the no-IMS samples ($\chi 2$ (2, N = 4) = 8.000, p = 0.018) have been removed with the result that no significant differences have been detected for the effect of temperature on *Giardia* DNA extraction from untreated stool samples stored for 3 months under the three temperatures of room, fridge, and freezer (Table 4.3). All three temperature conditions led to a loss of *Giardia* DNA within the three months (+ΔCt, Table 4.2).

Table 4.3: Effect of temperature on untreated samples.
Friedman test showed samples not treated with IMS to be significantly different at α= 0.5 level with a p-value = 0.018. They were therefore reanalysed with Bonferroni correction (p = 0.017) to attempt to pinpoint where the actual difference lies among the paired temperature conditions. If p value is larger than 0.017, the result is not statistically significant.

Temperature	Samples (with no IMS) tested on 4 different days in 3 months at 0.7-0.8 month intrevals (ΔCt)				Wilcoxon signed-rank tests (p) (2-tailed) with Bonferroni correction
	1	2	3	4	
Room Temp	9.5	9.1	9.5	9.9	0.068 (room/fridge)
Fridge	3.5	4.5	3.6	8.2	0.068 (room/freezer)
Freezer	3.1	2.9	0.4	4.1	0.068 (fridge/freezer)

4.3.2 Effect of IMS

Figure 4.2 shows the effects of IMS and the three temperatures on *Giardia intestinalis* DNA extracted from stored stool samples. IMS preserved samples had more DNA extracted from them than the untreated samples as indicated by the lower ΔCt values. Also colder temperatures slow down considerably the deterioration of *Giardia intestinalis* DNA indicated by the lower ΔCt values.

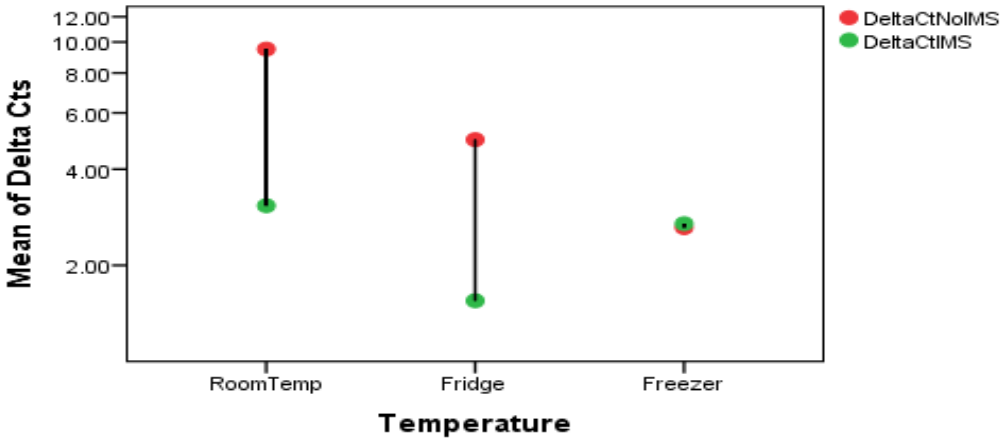


Figure 4.2: Effects of temperature and IMS on Ct values.
The ΔCts for the IMS treated samples stored at room temperature and in the fridge were lower than the untreated samples as can be seen from the mean ΔCts in Table 4.2 and illustrated in Figure 4.2. The differences in Cts (ΔCt) were also greater in samples without IMS stored at higher temperatures reflecting the increased rate of DNA deterioration. IMS made negligible difference to storage at -20 ºC (Figure 4.2).

The Wilcoxon rank sum test (similar to the Mann-Whitney test) which was used to evaluate the difference between medians for IMS-treated and untreated stool samples showed significant difference with $z = -2.656$, and $p = 0.008$. The mean of the ranks in favour of alcohol-treated stools was 8.67, while the mean of the ranks in favour of untreated stools samples was 16.33. The untreated stool therefore had comparatively less amount of DNA left at the end of the three months (the higher the Ct the less DNA there is to start with). Therefore at the level $\alpha = 0.05$ of significance, there was enough evidence to conclude that IMS reduced DNA losses or deterioration in treated stool samples.

4.4 Discussion

Stool samples contain many compounds that can degrade *Giardia* DNA and inhibit downstream enzymatic reactions. Also inappropriate storage conditions for these samples can adversely affect subsequent quality DNA yield for clinical laboratory diagnosis. Such a failure in methodology is likely to result in mis-diagnosed cases. I studied the effect of storage conditions for the preservation of stool samples and the extraction of *Giardia intestinalis* DNA.

As *Giardia* cysts are encountered more commonly in stools that are formed than in unformed stool and *Giardia* trophozoites encountered more in watery stool than in formed stool (Figure 4.3), a formed stool already seeded with *Giardia* cysts was used as the optimum sample for exploring storage conditions whilst minimising cost at the same time.

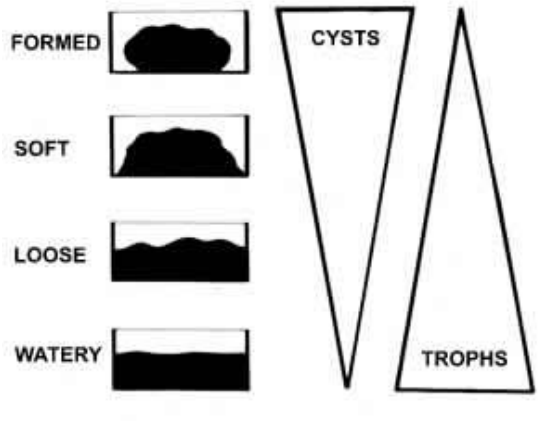


Figure 4.3: Distribution of protozoa in relation to stool consistency. Cyst numbers decrease from formed to watery stools whilst trophozoites numbers increase from watery to formed stools. (Source: http://www.dpd.cdc.gov/dpdx/HTML/Frames/DiagnosticProcedures/body_dp_stoolcollect.htm)

The influence of storage conditions on *Giardia* DNA detection from stool samples is discussed under the following headings:

1. The effect of time
2. The effect of temperature
3. The effect of IMS

In the analysis of these results, I attempted to find justification for either of the following two hypotheses: 1. The null hypothesis (H_0) which states that there is no significant difference in the DNA amounts expressed as delta Cts (ΔCts) being compared ($p > 0.05$), and 2, the alternate hypothesis (H_1), which rejects H_0 and states that there is a significant difference between matched pairs of results ($p \leq 0.05$).

1. <u>The effect of time</u>

There is a degree of degradation of DNA in stool samples during storage and as shown in Table 4.2, the ΔCts over the three month period showed unpreserved stool samples at room temperature to be the worst affected among three temperatures with a ΔCt of 9.5 for the untreated stool sample (i.e. no IMS added) and ΔCt of 3.1 for the treated sample (with IMS added). Storage conditions, including time, cause cysts to rupture with the release of their DNA content into the faecal environment. The DNA is then subjected to hydrolytic or oxidative degradation which can be accelerated by the presence of nucleases produced by the bacterial flora in the stool sample as nucleases break down DNA. These results confirm the breakdown of *Giardia* cysts and the degradation of DNA that was released during storage and before the extraction procedures were performed on the stool samples. As stool samples contain a large number of bacteria, they are able to degrade DNA rapidly. This is particularly marked in dead parasites when normal cellular repair processes have ceased, endogenous endonuclease activity and spontaneous depurination can result in relatively rapid breakage of DNA strands. Studies on stool storage conditions used for *Giardia* molecular epidemiological studies conducted in different countries and by different research groups and which produced a relative success of the molecular analyses in the range of 10-80 % reported time in storage ranging from 2 months to 15 years (Wilke & Robertson, 2009).

As the deterioration and degradation of *Giardia* parasites and their DNA in storage is established, it is important also to determine the point in time in storage at which DNA breakdown significantly. Knowing the storage temperature, the type of preservative, and the point in time in storage will help establish better conditions for sample transportation for molecular analyses.

2. <u>The effect of temperature</u>

It is clear that samples kept at room temperature without IMS preservative had higher ΔCts, meaning lesser amount of DNA than those kept at fridge temperature and freezer temperature without IMS Table 4.2. This and the

findings of Cnops & Esbroeck (2010) suggest that long term storage of stool samples for *Giardia* DNA extraction may require fridge or freezing temperatures. They used real-time PCR to evaluate the influence of storage time and temperature on the detection of *Entamoeba dispar* and *Entamoeba histolytica* and demonstrated an improved DNA detection in frozen stool samples compared to freshly stored samples. Comparing fridge conditions with freezer conditions, there was a lot more DNA available (ΔCt 2.6) in samples frozen at -20^{0}C without IMS than there were in those stored at fridge temperature of 4-6 ^{o}C without IMS (ΔCt 5.0) Table 4.2. DNA is less susceptible to degradation under freezing conditions when cysts have not released their DNA content, which they are more likely to do under fridge conditions than freezer conditions in the absence of IMS.

So far, in the absence of IMS, degradation of DNA during storage increases from lowest to highest: from freezer (ΔCt 2.6) to fridge (ΔCt 5.0) to room temperature (ΔCt 9.5). However, the best conditions as indicated in (Table 4.2) was IMS preserved stool at ordinary fridge temperatures (4-6 ^{o}C) with ΔCt 1.6. Refrigerating IMS preserved stool samples at 4-6 ^{o}C was better than storing them with IMS at -20 ^{o}C (ΔCt 2.7). It is also better than room temperature with IMS added (ΔCt 3.1). This result agrees with the findings of Wilke and Robertson (2009) who reported the superiority of storing ethanol preserved stool samples at 4-6 ^{o}C after using microscopy and real-time PCR to investigate storage conditions for seven different samples over a period of three months (Wilke & Robertson, 2009).

In summary, over a period of three months, freezer conditions for storage of stool samples without IMS appeared to be better than fridge and RT conditions for the extraction of *Giardia* DNA. But with IMS added, fridge conditions appeared to be the best of the three with the lowest ΔCt value of 1.6 indicating the best DNA detection. But how significant are these observed differences? For the IMS treated samples there were no statistical difference in

the distribution of ΔCt values, meaning DNA detection was comparable in samples stored at all three temperatures, upholding the null hypothesis (χ^2 (2, *N* = 4) = 4.500, p = 0.105). This is quite different from the result of the untreated samples. The Friedman results of the analysis of the untreated samples indicated that there was a significant difference in the medians of the Cts (χ2 (2, N = 4) = 8.000, p = 0.018). But when this data was further analysed using the Wilcoxon matched-pairs signed-rank test and controlling for Type I error (false positive) across tests using the Bonferroni correction, the results indicated no significant difference in ΔCts between the three pairs of room temperature/fridge (p = 0.068), room temperature/freezer, (p = 0.068) and fridge/freeze (p = 0.068) conditions (Table 4.3). The implication here is that the Friedman test result of (χ2 (2, N = 4) = 8.000, p = 0.018) was perhaps a Type 1 error which was corrected by the Bonferroni adjustment using a stringent p-value of 0.017 instead of 0.05 to determine significance levels. The 0.017 value was obtained by dividing 0.05 by the number of comparisons (3 in this study). Therefore in the no-IMS samples, the differences expressed as ΔCts among the three temperatures for the three-month period were not statistically significant.

In practice, stool samples stored at these temperatures for three months would still be satisfactory for *Giardia* DNA extraction for use in molecular amplification studies. Colder conditions are better for storing samples for molecular amplification studies. Freezing and thawing, however, tends to rupture cysts with the release of their DNA content. This increases the vulnerability of the DNA to attack by nucleases or other agents. Freezing samples may limit bacterial growth and enzymatic activity, but it can also break a sizable proportion of *Giardia* cysts exposing their DNA content to degradative processes when the sample is brought to room temperature (Wilke & Robertson, 2009).

3. The effect of IMS

When IMS preservation was considered together with the storage temperatures, there was a statistically significant higher amount of DNA detected. As shown in Figure 4.2, ΔCts for the IMS treated samples at room temperature and in the fridge were below that of the untreated samples indicating much improved *Giardia intestinalis* DNA detection in samples stored in IMS. The Wilcoxon rank sum test, which was used to evaluate the difference between medians of ΔCt for IMS-treated and untreated stool samples, was significant, $z = -2.656$, $p = 0.008$. Therefore, at the level $\alpha = 0.05$ of significance, there was enough evidence to conclude that storage in IMS improved DNA detection. IMS therefore has a preservative effect on the cysts in storage. It is also to be noted that IMS appeared to have no effect on *Giardia* DNA yield at -20 ^{o}C (ΔCt 2.7) compared with the no-IMS samples (ΔCt 2.6) (Figure 4.2). What this means in practice is, it may not be necessary to add IMS to stool samples when storing at -20 ^{o}C but will be beneficial to add IMS when storing in the fridge (4-6 ^{o}C) or at room temperature. Despite the improvement in DNA detection when IMS is used, IMS can also have adverse affect on DNA integrity as shown in Figure 4.4. The DNA turns from a solid band on the gel to a smear when it is denatured by IMS Figure 4.4 (a) and (b).

(a) (b)

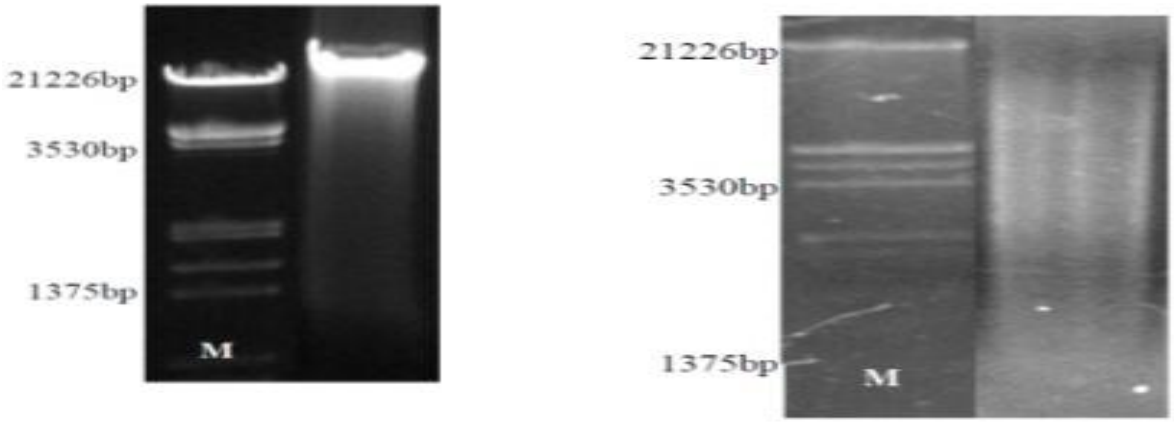


Figure 4.4: Gel electrophoresis showing the effect of IMS on DNA.
(a) whole dsDNA, (b) denatured DNA (Carter, 2003). M= molecular marker.

Methanol is closely related in structure to water and therefore can compete with water for hydrogen bonds. Consequently as a component in IMS, methanol can cause localized points of weakness in the dsDNA as a result of hydrogen bonding (Pearse, 1980). This effect notwithstanding, IMS does a lot more good than harm in the harsh environment of stool samples where DNA released from *Giardia* is subjected to hydrolysis, chemical, and enzymatic degradation. Extracted DNA from IMS preserved samples have been found to give positive results with both prokaryotic 16s and eukaryotic 18s PCR amplifications, and was digested by the RE enzyme Hinf1(Carter, 2003). This shows that even though the DNA was affected by the IMS, it was still good enough to be used to perform molecular analysis.

As a result, it appears evident that the most suitable storage condition, for stool samples to permit the isolation of *Giardia intestinalis* DNA among those investigated, was storage in IMS at 4-6 ^{o}C or frozen at -20 ^{o}C without IMS even though in the latter, there was very little difference in ΔCts between the IMS treated and untreated samples Figure 4.2. Under these conditions, stool samples may be stored for three months. Factors other than storage conditions can affect the successful amplification of DNA from stool samples. Cyst quantity, extraction technique, inhibitors, gene targeted, and choice of primers and cycling conditions can all impact on the success of molecular analyses. However, the use of optimum storage conditions is of paramount importance. The Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines is a set of reporting recommendations for authors of biospecimen-related research (Moore et al., 2011). They provide consistent and standardized information to better evaluate, interpret, compare, and reproduce experimental results (Moore, Kelly, McShane, & Vaught, 2012). The recommendations in the guidelines that this project addressed were on stabilization, preservation, storage temperature and duration of storage. The findings of this study support the case for BRISQ in the standardization of biospecimen-related research.

Chapter 5: Implementation of *Giardia intestinalis* real-time PCR

5.1 Implication for practice

After the demonstration of Kary Mullis' invention of the PCR technique on December 16, 1983, the technique has revolutionized the way clinical microbiology laboratories diagnose human pathogens. In clinical parasitology the OC&P method for a long time has remained the *de facto* gold standard for examining stools for parasites. Even though the method is time consuming and relatively less sensitive, it has remained popular and widely used because of its high specificity and the simultaneous detection of multiple parasites. There is, however, a good reason to look at alternate ways of examining stools for *Giardia intestinalis* as this research project has shown. Treating patients on clinical suspicion alone has had a measure of success in patients who diagnosed as microscopically negative for *Giardia*. If the parasites were present, but could not be seen using the microscope, then perhaps they were there in very low concentration or in a disrupted state and therefore a very sensitive test was what was required to confirm their presence in the sample examined.

In 1995 Low and McGeer published an article in New Horizon on the use of molecular biology techniques for diagnostic microbiology and hospital epidemiology. They predicted, in this paper, the development of molecular techniques for routine laboratory use in the "next decade" (Low & McGeer, 1995). The prediction has become a reality for the Department of Clinical Parasitology inasmuch as molecular techniques for the diagnosis of microsporidia (Polley et al., 2011) has replaced microscopy and the case for a real-time PCR to replace OCP-M for *Giardia* infections has been explored in this project (see a summary of the achieved objectives in Table 5.1).

Table 5.1: A summary of achieved objectives for this study.

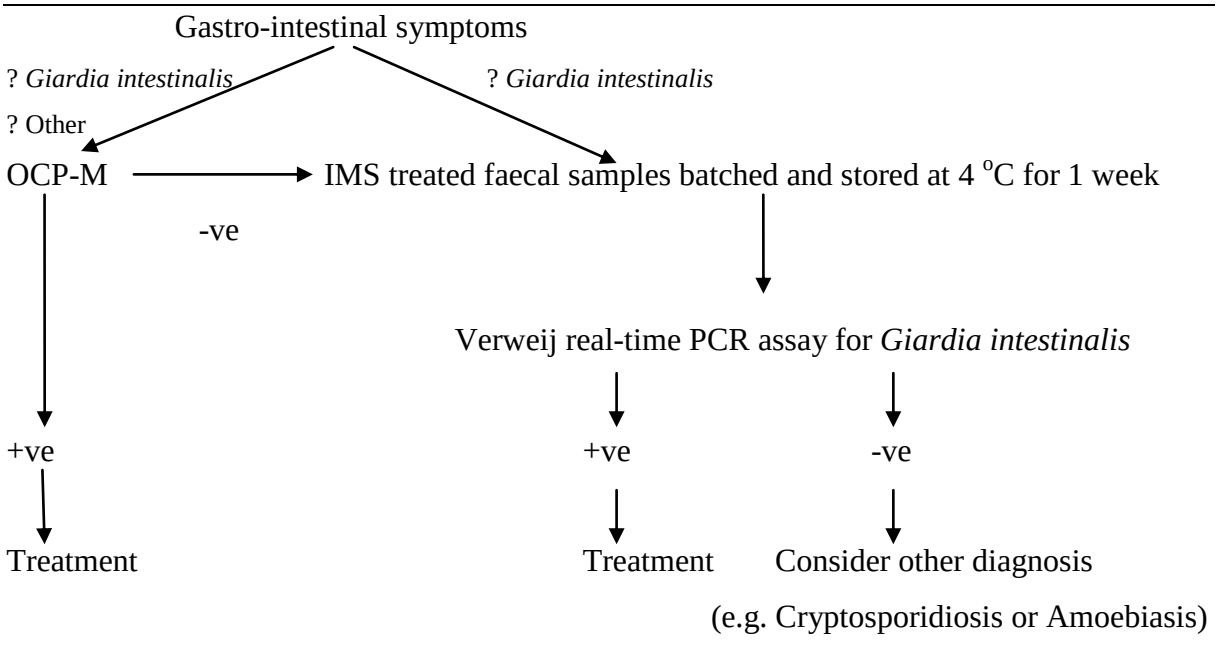
Objectives	Description
01	To carry out an exploratory investigation using spiked stool samples to determine any differences in performance between the index tests: Ova, cyst and parasite-microscopy (OCP-M) and real-time PCR.
01 Achieved	The Verweij real-time PCR had a p-value of < 0.05 for performance and a limit of detection that was even lower than the assay parameters for this study (< 5 cysts/ml of stool)
02	To carry out an exploratory investigation using spiked stool samples to ascertain the analytical sensitivity and specificity of the reference tests: EIA (Enzyme immunoassay) and rapid membrane test (RMT).
02 Achieved	The limit of detection of $\leq$ 2840 cysts/ml and $\leq$ 92 trophozoites/ml determined the strength of detection of the composite reference standard.
03	To evaluate and compare the accuracy measures for OCP-M and real-time PCR using a non-probability sampling technique with consecutive samples.
03 Achieved	See Table 3.7 for tabulation of all the results. The Verweij real-time PCRhad the highest sensitivity of 93.4 %.
04	To compare the accuracy measures for real-time PCR to that of a combination of OCP-M and real-time PCR.
04 Achieved	The sensitivity remained unchanged at 93.4 % following the amalgamation of the two tests.
05	To investigate the effect of different storage conditions on the detection of *Giardia intestinalis* DNA in human stool samples.
05 Achieved	IMS preserved stool at 4-6 ^{o}C for three months appeared to be the best condition for keeping stools before DNA extraction.
06	To develop an algorithm and a business plan for use in the Department of Clinical Parasitology for the laboratory investigation of giardiasis incorporating real-time PCR.
06 Achieved	See Figure 5.1 for the algorithm and Appendix V for the business plan.

The information provided in the section that follows form part of a business plan (see Appendix V) I have written up to introduce the Verweij real-time PCR into the Department of Clinical Parasitology at the HTD in London. In this section 5.1, the algorithm to be followed and the pricing of the test consequent on the adoption of the real-time PCR into routine practice are presented. For a wider discussion of the business plan which includes service delivery, research and development, see Appendix V. This Section 5.1 and the business plan dossier in Appendix V address objective 06 of this study.

5.1.1 Algorithm

The following algorithm is recommended for the laboratory diagnosis of giardiasis (Figure 5.1).

Figure 5.1: Algorithm for the laboratory diagnosis of giardiasis.



+ve = Positive for *Giardia intestinalis*; -ve = Negative for *Giardia intestinalis*.

Microscopy will still be useful as part of differential diagnosis but should not be relied upon in all post treatment follow up investigations. This algorithm has an important step that was discovered in this research project and it is the addition of IMS to stool samples before DNA extraction. The IMS treated samples are to be stored at 4-6 ^{o}C and used for *Giardia* DNA extraction.

5.1.2 Pricing

The running cost per test for the Verweij real-time PCRwas worked out according to Table 5.2 consumables (£6/test) and Table 5.3 labour costs (£331/test).

Table 5.2: Cost of key consumables of the Verweij real-time PCRassay.

Item	Price per pack (£)	No. of tests performed per pack	Cost per test (£)
Direct costs			
Primers & Probes (Working solution) **(a)**	209.40	300	0.70
Environmental mastermix **(b)**	565.00	400	1.40
QIAamp DNA mini QIAcube Kit **(c)**	1576.80	480	3.30
Pastettes **(d)**	10.00	500	0.02
Extended Fine tip pastettes **(e)**	17.20	250	0.07
1.5 ml Eppendorf tube **(f)**	26.31	1000	0.03
Sarstedt tubes **(g)**	15.50	500	0.03
Sarstedt tube caps **(g)**	24.90	500	0.05
		Total:	5.60 (~6.00)

Keys (manufacturers):

(a) Eurofins **(b)** Applied Biosystems **(c)** Qiagen ltd., **(d)** Elkay Precision Laboratory **(e)** Alpha Laboratories **(f)** Eppendorf, **(g)** Apex.

Table 5.3: Labour cost for multiplex protozoal PCR.
This test incorporates the Verweij *Giardia* real-time PCR and the BMS band 7 mid-point salary of £35,184 p.a. (1/4/2011 pay band) was used in the calculation.

Sample processing (£)	Time taken (min)	BMS Band 7 salary per minute (£)	Cost
Washing	240	0.30	72
Lyses	384	0.30	115.2
Extraction	240	0.30	72
Real-time PCR	240	0.30	72
		Total:	331.2

This provided the running cost per test as £337. This approach was taken since there is no competition currently. The amount is heavily subsidized due to the fact that additional equipment and materials required to run this test are available and already catered for under the departments purchases for PCR work. The running cost per test (£337) for *Giardia* investigations is equivalent to that for *Cryptosporidia* and *Entamoeba histolytica* in a multiplex reaction and so requesting *Giardia* PCR has the other two PCRs added on as well. The current listed price (2013) for requesting a protozoal PCR for any of the three parasites is £53 for National Health Service patients and £68 for private patients.

5.2 Further research

1. One of the strongest reasons to continue to use OCP-M is that other parasites apart from *Giardia intestinalis* are detected. Multiplexing the Verweij real-time PCR to include other diarrhoea causing parasites like *Cryptosporidium*

and *Entamoeba histolytica* has been done and it will be good to widen its scope to include other parasites producing similar clinical symptoms (e.g. *Cyclospora*) and possibly also parasites across the genus divide of bacteria and viruses. PCR based diagnostic tests have arrived and the modernisation and formation of joint ventures between pathology services will be the driver for the increased use of it in diagnostic laboratories because of the multiplexed tests for panels of gastrointestinal pathogens (R.M. Chalmers, 2009).

2. Further research will be needed to improve upon the analytical sensitivity of the Verweij real-time PCR using *Giardia intestinalis* trophozoites and to optimise the assay for non-faecal samples like duodenal aspirates and biopsies where trophozoites of *Giardia intestinalis* are likely to be encountered, and also for pus or liver aspirates in amoebic abscess cases where *Entamoeba histolytica* are likely to be encountered in multiplex reactions.
3. Further research is required to optimise the Primerdesign Ltd. real-time PCR kit for use in longitudinal surveillance and genotyping of positive cases of giardiasis. The use of this kit will provide more information on the potential public health risk from domestic dogs and cats and the frequency of zoonotic *Giardia* transmission.
4. There is the need for large-scale molecular epidemiological surveys of *Giardia* infections in humans to determine the geographical distribution and prevalence of human-infective genotypes. This type of information can also shed light on asymptomatic giardiasis as to whether it is “assemblage”-related or not.

5.3 Conclusion

1. OCP-M, with a sensitivity of 83.5 %, failed to detect 16.5 % of true giardiasis cases in this study. Similarly, with a sensitivity of 93.4 %, the Verweij real-time PCR failed to detect 6.6 %. The Verweij real-time PCR

therefore diagnosed approximately 10 % more giardiasis cases than the OCP-M and 19.3 % (94.3 - 75 = 19.3) more positive cases with adjusted sensitivities.

2. The Verweij real-time PCR has been shown to be a more efficient and robust diagnostic test for use as a first line test for giardiasis. Compared with the OCP-M, the Verweij real-time PCR correctly diagnosed 70 % (14/20) of the discrepant cases as giardiasis and the OCP-M correctly identified only 10 % (2/20).

3. The Verweij real-time PCR can be used as a standalone test (if it needs be) for the diagnosis of giardiasis. In combination with the OCP-M, there was no improvement in sensitivity over the Verweij real-time PCR when compared with the latter alone. The sensitivity remained at 93.4 %. OCP-M, however, has the advantage of identifying the presence of other parasites.

5. With LOD < 5 cysts/ml, the requirement for the submission of three stools for the diagnoses of giardiasis or for post treatment follow up investigation is now non-critical as a result of the robustness of the Verweij real-time PCR.

6. Storage of stool samples in IMS at 4-6 ^{o}C prior to DNA extraction for *Giardia intestinalis* was the best storage condition among those investigated.

The aim of this thesis has been to investigate the possibility of deploying a non-microscopy based test (i.e. Real-time PCR) as a frontline test for the diagnoses of giardiasis. The Verweij et al. (2003, 2004) real-time PCR provided a worthwhile platform for this study and has shown a lot of potential over the OCP-M that is currently in use. In patients with suspected giardiasis, the use of the Verweij real-time PCR will lead to improved detection of *Giardia intestinalis* in their stool samples.

Even though the Verweij real-time PCR has proved to be a very sensitive diagnostic tool for testing faecal samples for *Giardia intestinalis*, it has some

limitations and the recommendations given below, when followed, will improve upon the performance and turnaround times of the assay.

Recommendations:

1. IMS should be added to stool samples meant for *Giardia intestinalis* DNA extraction.

2. It takes a long time to wash the stool samples, 3–4 h or even more depending on the number in order to get them ready for the lyses stage which then requires overnight incubation. Automated washing and extraction devices will speed up these essential first steps in the real-time PCR protocol. Extra investment in equipment to automate the process will however be required.

Chapter 6: Reflection

6.1 Introduction

The final chapter of this thesis heralds the end of the study of a well-put together study programme that, for me, has been fantastic and very rewarding in every way. I will be reflecting on this experience shortly, after this brief account of an episode of giardiasis I had over three decades ago which gave me the passion for this project and the reason to be curiously interested in it.

A personal experience I had with giardiasis took place towards the latter part of the 1970s when I was training to be a multi-disciplinary medical laboratory technician in Accra, Ghana. For a period of time, I experienced irregular bowel movement which later gave me a skin rash with mild itching sensations. Normal bacterial cultures at the time yielded nothing and I was asked to submit a stool sample for ova, cysts and parasite examination. It was then that I was diagnosed with giardiasis. I had the opportunity to look down the microscope myself and saw the intestinal flagellates that Antony Van Leeuwenhoek saw in his stool 332 years ago in 1681. The intestinal flagellates were *Giardia intestinalis*. I was treated with Metronidazole and got better. Despite the fact that my experience with *Giardia* was not the most pleasant moments in my life, it gave me the impetus to undertake this project with greater interest.

6.2 Critical reflection of the learning

During the first year of this doctorial study in 2008/9, I justified the use of Gibbs' reflective cycle when I reflected on my previous learning before enrolling on this course. Reflective practice was then a new area of study for me and Gibbs' model was less complicated to follow at the time. I have decided to use a different model now to critically reflect on my learning as this study program draws to the end. Reid (1993) has been quoted as saying that reflection

is “a process of reviewing an experience of practice in order to describe, analyse, evaluate and so inform learning about practice”(Bulman & Shutz, 2004). With that in mind, the model I have, therefore, chosen is the Atkins and Murphy’s model of reflection (Atkins & Murphy, 1994). This model (Figure 6.1) provides a useful questioning framework for reflection based on the definition given above.

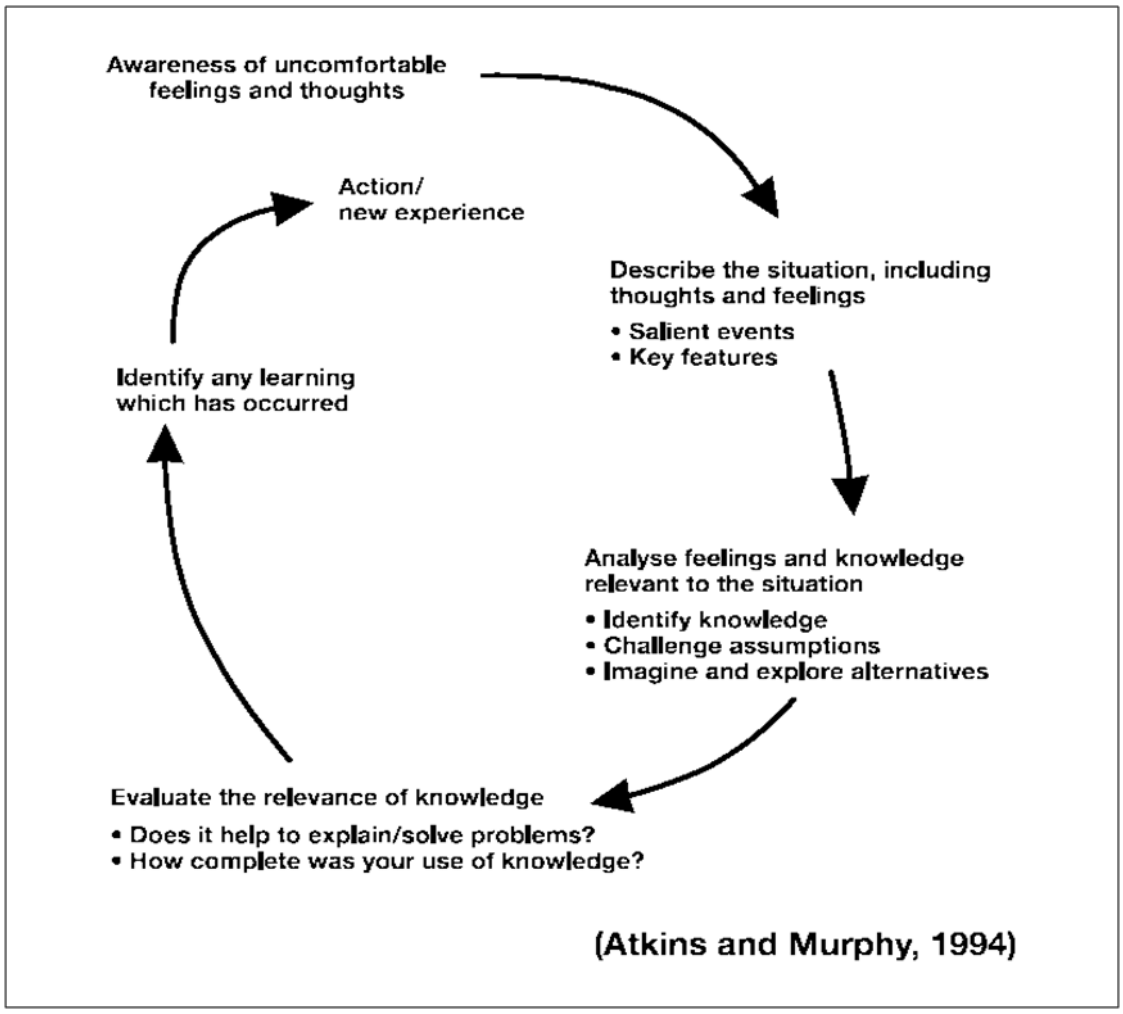


Figure 6.1: Atkins and Murphy model of reflection (1994).

An example of how the cycle works is given briefly below using the audit for service improvement study I did years ago before enrolling on this course. I am using it to illustrate how the process of reflection works with this cycle. The example relates to a very specific incident and truly forms part of my overall experience on this course. The fact that I have extracted this experience from

the big picture to illustrate how the reflective cycle works, does not mean that this incident was not directly related or relevant to my overall experiences on this course.

Awareness of uncomfortable feelings and thoughts

Due to staff shortages, I spent an unusually long time on the enteric bench in the microbiology department. The rota for moving from bench to bench was not working properly and as a result, I became tied and fed up for been in one section for so long.

Describe the situation, including thoughts and feelings

One particular aspect of the work in this section was tedious and that made me unhappy. It was concerned with a particular screen profile (acid and gas only producers in triple sugar iron agar) that suggested a pathogen (*Salmonella* or *Shigella species)* but most of the time produced negative results upon further lengthy investigations. I thought to myself to find a way of looking into this testing procedure in order to introduce a test that will be less laborious to perform in the least amount of time. When I shared this with my section head/ laboratory manager, I was given the permission to proceed with the work. I collected about seventy one of the isolates that fitted this profile (see above) and searching through the literature found a test for β-galactosidase activity and used it over a series of timed periods to classify these isolates on the bases of the production of the β-galactosidase enzyme. The latter determines the ability to ferment lactose and *Salmonella* and *Shigella* with very little exception are known as non-lactose fermenters. 67 % of the isolates which produced Beta-galactosidase also broke down the substrate within 30 min and therefore they were classified as non-pathogens. This test is now used routinely as a result of my work.

Analyse feelings and knowledge relevant to the situation

I felt a sense of accomplishment that I have made the identification procedure more efficient by challenging the status quo and successfully

exploring alternatives. What I did not have then was the no-how of publication and dissemination of research information to enable me publish a paper on this work.

Evaluate the relevance of knowledge

Even though I had the technical expertise to investigate and solve this problem, my knowledge was not complete, in that publication and dissemination were lacking in my experience.

Identify any learning which has occurred

I acquired a deeper understanding of the diagnostic procedure to a level that only comes by experience (Kolb, 1984) and which is higher than Bloom's taxonomy level two (Anderson, Krathwohl, & Bloom, 2001). I needed to publish as expected of research scientists.

Action / New experience

I enrolled on this Professional Doctorate's course and as a result had the experience of preparing a paper on this piece of work for publication. The paper was accepted and has since been published in the Journal of Clinical Pathology under the title: Selective testing of β-galactosidase activity in the laboratory identification of *Salmonella* and *Shigella* species (Boadi, Wren, & Morris-Jones, 2010). The process of reflection has gone full cycle and it begins again with the new experiences that come with studying at doctoral level.

This is the format that I will now use to analyse my learning over the past 4-5 years since October, 2008 when I enrolled on this course.

I will also be relying on the "Cake mix" method of keeping portfolios to reflect on my learning experiences. The "Cake mix" model provides an overarching narrative of one's learning journey with the main features of this model being reflectivity, practice and professional development (Endacott et al., 2004).

The timeline of my experiences showing the relative level of satisfaction in percentage (%) at any stated point in time is shown in Figure 6.2. This timeline illustrates what is being discussed.

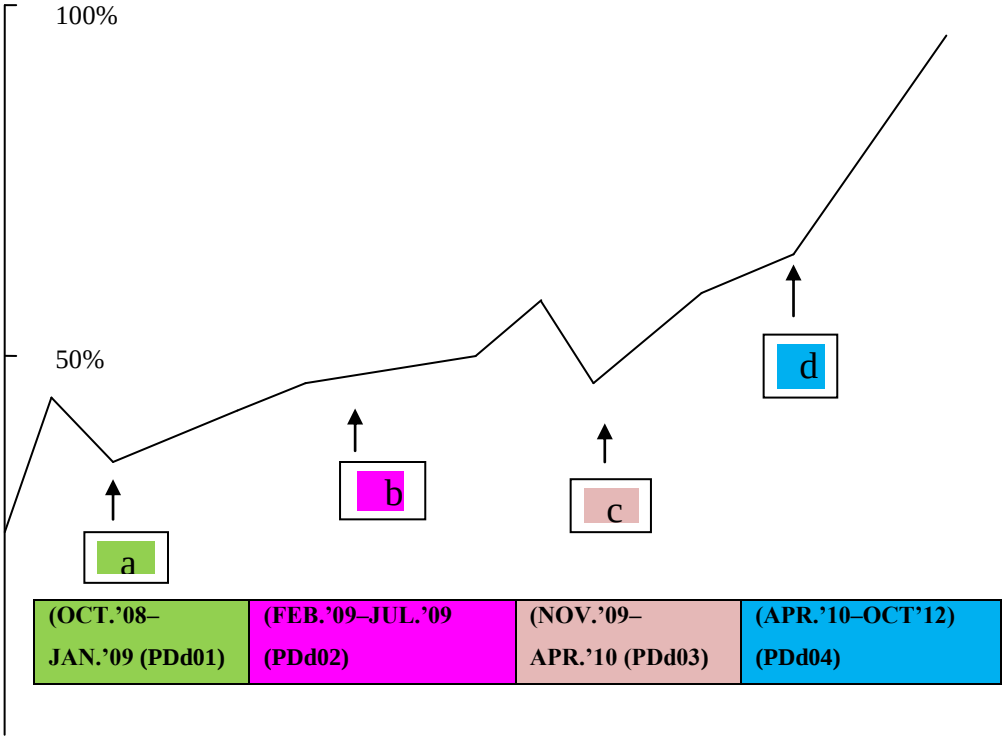


Figure 6.2: Timeline of my experiences.
The relative level of satisfaction is shown as percentage (%) at any point in time.

Awareness of uncomfortable feelings and thought:

Less than a month into this professional doctorate course, I wrote in my online journal on the 8th of November, 2008 the following excerpt after I have had my first ever lecture on reflective thinking and writing about ten to twelve hours earlier. Unaware that it had already gone midnight, I wrote:

"Date: 08 November 2008 00:03

Today's lecture on the subject above has revealed how very little reflective writing & thinking I have been doing in my professional life so far.... I am engaged in a lot of CPD activities but have not been doing any reflective writing or thinking about them.

I need to think critically and do reflective writing from now onwards if I am going to be an expert in my area of practice. This area of learning is new to me. It was not part of the curriculum for my previous studies and it is a challenge that I have to take on now".

This experience captures the “awareness of uncomfortable feelings and thoughts” that Atkins and Murphy (1994) put as the first step in their model of reflection.

Describe the situation, including thoughts and feelings:

OCT.’08 – JAN.’09 (PDd01)

The awareness of uncomfortable feelings and thoughts triggered by the reflective writing/critical thinking lecture, made me question myself whether I can succeed in the studies at doctoral level. It was just a feeling at that early stage but an uncomfortable one at that. This was a low moment for me (Figure 6.2 arrow [a]) about three weeks into the study of this unit haven started this course with high hopes of succeeding. I later on read a review of the NHS Knowledge and Skills Framework (NHS KSF) (Standards of proficiency - Biomedical scientists, 2012). It discussed the relevance of the Government’s aim to achieve strong and educated workforce in the NHS and this also generated further impetus for my personal development. It helped me to set and work on one of the principal objectives of the unit, PDd01, which was to identify, manage, and demonstrate a reflective and self critical approach to the application of learning to one’s professional practice. The PDd01 unit was completed on 23rd January, 2009 and by that time my satisfaction level was rising again from the decline it suffered earlier on when the unit started (Figure 6.2).

FEB.’09 – JUL.’09 (PDd02)

The Advanced Research Technique (ART) unit, PDd02, began with an assessment of research skills. The areas of low confidence levels for me were e.g. the search for research papers online. This was resolved with formal lectures and the online studies. I also undertook an Epigeum course on project management and I completed it successfully. These courses together with the appraisal of quantitative and qualitative research exercises and the statistics course helped me a great deal to put my research guide exercise for this unit

together successfully. I was one of the only two people who passed the first time without re-submitting it. My satisfaction level was on the ascendency during the course of this unit. This satisfaction was also bolstered by the identification of a service improvement audit at my work place. With the help of other staff members, I successfully carried out this audit to justify the need to bring in a new diagnostic test for the diagnosis of microsporidial parasites.

NOV.'09 – APR.'10 (PDd03)

I have enjoyed the course so far and the graph was on the ascendency throughout the ART/PDd02 unit (Figure 6.2 arrow [b]) to the middle of the Publication and Dissemination (PDd03) unit when my level of satisfaction dipped again (Figure 6.2 arrow [c]) because an article I wrote as a class exercise was improperly handled by a colleague who peer reviewed it. I am happy to report that the article was subsequently reviewed by two experienced work colleagues and has since been accepted and published by the Journal of Clinical Pathology. This was the article (selective testing of β-galactosidase activity) that was mentioned above when Atkins and Murphy's 1994 model of reflection was illustrated. My level of satisfaction rose again when the article was accepted for publication (Figure 6.2 arrow[d]).

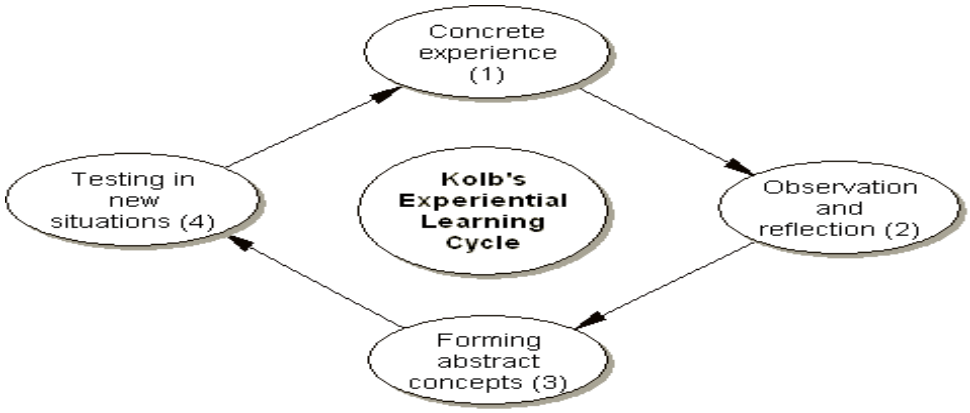
APR.'10 – JUL.'11 (PDd04)

My satisfaction level has continued to rise. The latest addition to that was the passing of my project proposal (PDd04) at the first attempt without re-submission. The above are the salient events/key features as I have been on the course so far. I will now proceed to analyze these events.

Analyse feelings and knowledge relevant to the situation:

The Part One studies has been a thorough and worthwhile experience for me. I have acquired thinking, writing, and presentation skills that are fundamentally important for this level of academia/professional life. What is even more exciting for me is that these skills have all been used in the studying and application of the advanced research techniques unit (PDd02). Ultimate

learning was acquired through exposure and by actually practicing the use of these skills in real life scenarios. This brings into focus Kolb's experiential learning theory. Kolb defines learning as "The process whereby knowledge is created through the transformation of experience"(Kolb, 1984). Kolb's experiential learning theory is a holistic perspective that combines experience, perception, cognition, and behaviour. The theory presents a cyclical model of learning, consisting of four stages: Concrete experience (DO), reflective observation (OBSERVE), abstract conceptualization (THINK) and active experimentation (PLAN). Any of the stages can be a potential starting point for the cycle (Figure 6.3).



.

Figure 6.3: Kolb's learning cycle.

Looking at the big picture, my concrete experience began when I ***did*** enrol on this course and through the reflective writing/critical thinking lectures I ***observed*** the importance of this area of study and used it to ***think*** back in my professional life and also think ahead into the future. Specifically in the area of publication and dissemination I became acutely aware of the need to publish. I put a plan together and wrote an article for publication. The learning experience has been holistic and exciting and in every sense beneficial to my professional life. Kolb also identified four learning styles which correspond to these stages. The styles highlight conditions under which learners learn better. These styles are: assimilators, who learn better when presented with sound logical theories to consider; convergers, who learn better when provided with practical

applications of concepts and theories; accommodators, who learn better when provided with "hands-on" experiences; and divergers, who learn better when observing and collecting a wide range of information. As much as I agree with the sentiments of Kolb's experiential learning cycle, I find it difficult to comprehend how these four learning styles could be compartmentalised in one's experiences. For me, I can identify with all four styles at one time or another in life/professional experiences.

Evaluate the relevance of knowledge:

Learning is a major process of human adaptation to new situations and throughout this course, it has entailed a great deal of sacrifice and self discipline in the midst of life's challenges. The endurance of any building enterprise, including that of life, relies very much on the strength of its foundation and life experiences. These experiences could arise from a personal study project or a totally unplanned occurrence in daily life (Boud, Keogh, & Walker, 1985). It could be initiated by an external or personal interest and will have smaller experiences involved, a kind of a wheel in a wheel effect.

Identify any learning which has occurred:

The learning which has occurred now is the acquisition of reflective writing and critical thinking skills, presentation skills, oral as well as written, publication and dissemination skills, and advanced research techniques for searching for and using information.

Action/New experience:

As a consequence of the learning which has occurred, the new experience of a leadership role is beckoning and I think will be demanded on successful completion of this course. This will be the potential new experience that re-starts the model of reflection.

6.3 What has the Professional Doctorate done for me?

As I reflect on my experiences, I am profoundly amazed at the depth of understanding that I have acquired in my professional life as a result of embarking on this programme of studies. I am confident that I have displayed originality in using my knowledge base and methods of inquiry in practice and hence have addressed the specific learning outcome which requires students to instigate theory of quantitative research methodologies with professional expertise to propose strategies to solve problems relevant to health.
In the process, I have developed some contacts at the LSHTM and through networking have progressed in my professional and academic development.

One significant improvement since I have been on this course is in the area of publishing. I had no experience at all in publishing when I enrolled on this course and since the publication and dissemination unit, I have authored and published a paper and have also been co-author of two other articles also published. I have also reviewed two articles submitted for publication in the Journal of Clinical Pathology. All these publication activities (listed below) together with reflective practice have enabled me to be a confident practitioner, able to help trainee biomedical scientists to develop these skills as well.

Publications activities

1. First author:

Published

Boadi, S., Wren, M. W., & Morris-Jones, S. (2010). Selective testing of ß-galactosidase activity in the laboratory identification of *Salmonella* and *Shigella* species. *J Clin Pathol, 63*(12), 1101-1104.

2. Second author:

Published

Polley, S. D., **Boadi, S.**, Watson, J., Curry, A., & Chiodini, P. L. (2011). Detection and species identification of microsporidial infections using SYBR Green real-time PCR. *J Med Microbiol, 60*(Pt 4), 459-466.

3. Co-author:

Published Open Access Research

Roberts, C. H., Armstrong, M., Zatyka, E., **Boadi, S.**, Warren, S., Chiodini, P. L., et al. (2013). Gametocyte carriage in *Plasmodium falciparum*-infected travellers. *Malar J, 12*(1), 31.

4. Reviewer:

a. Reviewer of manuscript # jclinpath-2011-200127 entitled "When histopathology is not needed: Macroscopic identification of botfly *Dermatobium hominis*) infection." for Journal of Clinical Pathology.

b. Reviewer of manuscript # jclinpath-2011-200403 entitled "Forgotten opportunistic parasitoses emerging in patients receiving alemtuzumab" for Journal of Clinical Pathology. Published on 3/11/2011 as: “Two cases of opportunistic parasite infections in patients receiving alemtuzumab”

Not only in the area of diagnostics did I experience advancement but also I gained a modicum of business acumen during my project work. I communicated with customer services of the manufacturer’s of the items I used in my project and by that have gained more experience in my communication skills. Putting together the business plan was a new experience and I had to learn new terminologies used in the business world (e.g. direct cost and labour cost).

6.4 What has learning been for me?

Learning for me has been experiential, the equipping and the application of knowledge refined through dialogue and networking with other professionals. The Advanced Research Technique unit enabled me to put together a research

guide and my project proposal. The Publication and Dissemination unit enabled me to publish work done. Reflective practicing and critical thinking enabled me to review my work and make the necessary changes that ushered in progress. All together, the professional doctorate course has given me the tools I need for advancement in my career and life in general. Albert Einstein has been credited with the saying: "Insanity is doing the same thing, over and over again, but expecting different results". As I draw to a close, five years of new experiences in my life, I can say that the truth of Albert's statement resonates with my life. I am not where I hope to be yet, but I can truly say that I am not where I used to be.

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Confidential information has not been disclosed in the appendices but where information is already in the public domain a reference to assess it has been provided.

Appendix I: C-Chip counting chamber
(PEQLAB Ltd., n.d.).

Appendix II: *Giardia*-Strip kit insert
(Coris BioConcept, 2012).

Appendix III: Techlab EIA kit insert
(TECHLAB, 2006).

Appendix IV: Primerdesign Genesig Advanced kit handbook
(Primerdesign Ltd., n.d.).

Appendix V: Business plan. HTD Department of Clinical Parasitology (Confidential report)

Appendix VI: UltraClean 15 DNA Purification Kit (From agarose gels and solutions) –Instruction manual
(Mo Bio Labs, 2010).